Investigation of effects of the toxic cyanobacteria *Microcystis aeruginosa* on zebrafish reproductive system physiology at molecular, tissue and whole organism levels of biological organization

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ABSTRACT

Blooms of toxic cyanobacteria in freshwater bodies are a global ecological and public health concern. Microcystin (MC) toxin concentrations can exceed the recommended levels of the World Health Organization. Among microcystin (MC) toxin types, MC-LR is generally accepted as the most toxic congener and the one most considered in research. MCs have been associated with fish mortality and the sublethal effects of aqueous and dietary exposure on larval and adult zebrafish (*Danio rerio*) at environmentally relevant concentrations are relatively unknown.

The objectives of this research project were novel. First of all, to investigate the toxicity of *M. aeruginosa* and MC-LR in zebrafish larvae using gene expression profiles and histopathology with dose and time response. A further objective was to investigate the effects of sub-lethal dietary exposure of both the cyanobacterium, *Microcystis aeruginosa* and the MC-LR toxin on adult zebrafish, through evaluations of target gene expression, changes in the gut microbiota and histopathology.

The results of the sub-lethal aqueous exposure of MC-LR and *M. aeruginosa* for 96 hours on zebrafish larvae indicated oestrogenic effects for MC-LR and *M. aeruginosa*. Significant changes in gene expression were observed according to treatment concentration and exposure duration for oxidative stress and biotransformation related genes. However, treatment-related changes in gene expression did not relate to hepatocellular or intestinal lesions in larval zebrafish.

Results after the sub-lethal dietary exposure of adult zebrafish to MC-LR or *M. aeruginosa* for 14 days increased significantly for the target genes depending on treatment and exposure duration. Additionally, no histopathological changes in the liver or the gut were observed, apart from normal variations. However, the trunk kidney showed indications of karyorrhexis, hypertrophy and hyperplasia. Moreover, changes in gut microbial community structure were recorded.

Finally, the results of the present study showed changing gene expression profiles for different target genes at dose and time relationships after exposure to the sub-lethal aqueous and dietary exposures. Such exposures also suggested that oxidative stress could be another toxic route along with the protein phosphatase pathway. Changes in the gut structure imply that effects on gut physiology could in addition be an important toxicological response to *M. aeruginosa*.

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Alawchi, Sabah N.; Alkasiy, K.S. and **Sabrei, D. A.** 2009. Study the Effect of Thyroxin and the Aqueous Extract of Borage (*Borago officinalis*) on the Activity of Alkaline Phosphatase (ALP) in Neutrophils in Male Albino Mice. *Iraqi Journal for marking research and protect the consumers*, 1 (2).

Alawchi, Sabah N.; Alkasiy, K.S. and **Sabrei, D. A.** 2009. Study the Effect of the Aqueous Extract of Borage (*Borago officinalis*) on the Function and Structure of Thyroid Gland in Male Albino Mice. *Iraqi Journal for marking research and protect the consumers*, 1 (2).

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Chapter One

Introduction

1. Introduction

1.1. Harmful algal

Harmful algal blooms can produce toxins that are a major public health and environmental concern in surface waters around the world (Merel et al., 2013; Boopathi and Ki, 2014). Although strictly not algae (which are eukaryotic), cyanobacteria (which are prokaryotic) also form blooms that are of particular importance because toxin concentrations regularly exceed levels considered safe for human, wildlife and aquatic ecosystem health. Since the late 18th century, when the first blooms of cyanobacteria were described in Australia by George Francis, blooms of cyanobacteria have been reported in many freshwater bodies around the world. The formation of blooms of cyanobacteria may be accelerating because of rapid global eutrophication of fresh water (Abdel-Rahman et al., 1993; Jacoby et al., 2000).

An algal bloom is a rapid increase in the population of phytoplankton algae in an aquatic system (Figure-1-1). Usually, just one or few species are involved in making the bloom. The bloom is recognised through discoloration of the water which is a consequence of the high density of pigmented cells. The threshold number of algal cells to consider as the bloom is more than 10,000 cells/ml (Florczyk et al., 2014). Algal bloom concentrations may reach millions of cells per milliliter. Different colors are observed during the blooming process such as green, yellowish-brown, red or bright green (Chen et al., 2009; Florczyk et al., 2014). Blooms affect the water quality, especially drinking water because they can produce substances such as two methylisoborneol and geosmin which cause unpleasant tastes and odors in water (Chen et al., 2009; Florczyk et al., 2014). In addition, blooms can also change water pH, transparency, biodiversity and produce a variety of toxins including microcystin, nodularin and anatoxina (Li et al., 2011). The harmful algal blooms could link to overfeeding through overloading of nutrients, which are mainly phosphorus, nitrogen and carbon from different sources such as lawns and farmlands (NOAA, 2014).

A B

Figure 1-1: A: *Microcystis* blooming. B: *Microcystis* bloom in a Scottish freshwater lake (NOAA websites, 2014) / http://www-cyanosite.bio.purdue.edu/images/images.html

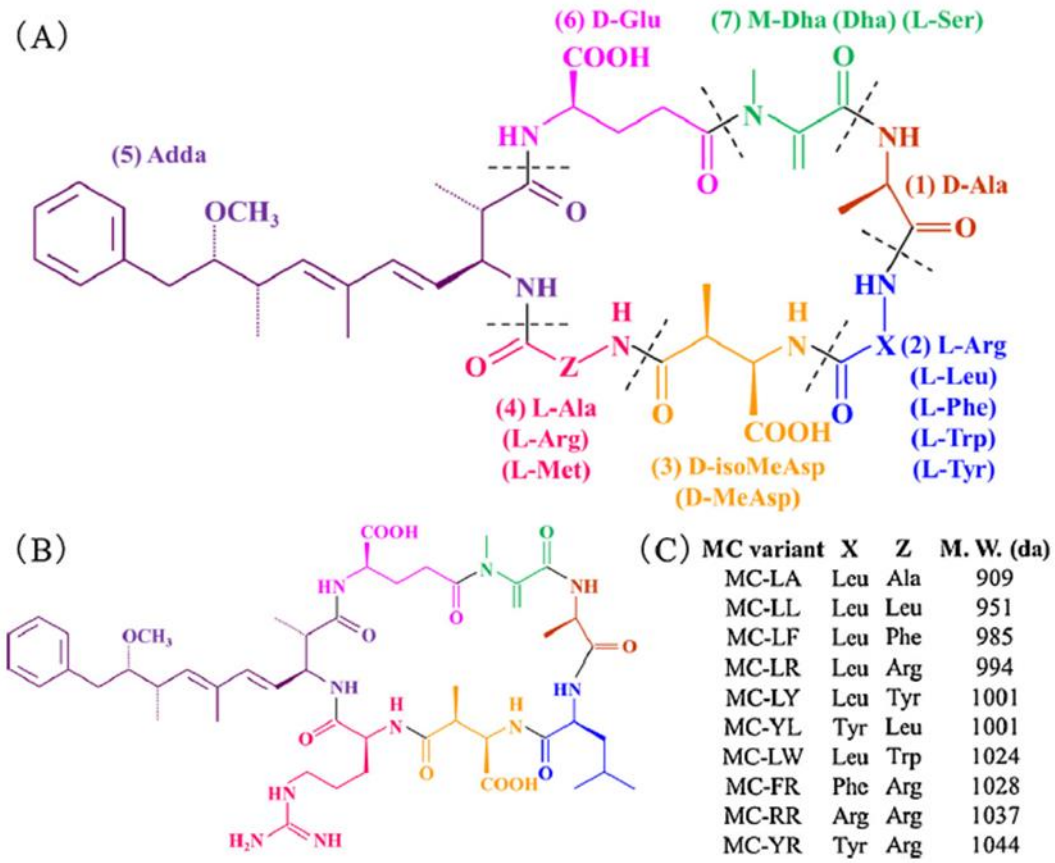
1.2. Cyanobacteria characteristics

Cyanobacteria are among the earliest organisms on Earth and can release oxygen to the atmosphere via photosynthesis (Mur et al., 1999). Cyanobacteria "blue-green algae" are prokaryotic bacteria that include many species such as *Microcystis* spp., *Anabaena* spp. and *Cylindrospermopsis spp.* that produce a variety of toxic secondary metabolites which are called cyanotoxins (Campos and Vasconcelos 2010; Dittmann et al., 2013). Cyanotoxins are generally divided into five classes based on their target organ, First of all, hepatotoxins ct on the liver (such as microcystins and cylindrospermopsin). Second, neurotoxins act on the nervous system (such as saxitoxin and anatoxin-a). Third and forth, cytotoxins and dermatotoxins which cause skin irritation (such as lyngbyatoxin). Finally, endotoxins (such as lipopolysaccharides), which cause a headache, fever, gastrointestinal illness, skin rashes, allergy and respiratory disease (Codd et al., 2005; Kuiper-Goodman et al., 1999; Jaja-Chimedza et al., 2012).

1.3. The chemical structure of microcystin toxins

Microcystins (MCs) toxins are toxic cyclic peptides with seven amino acids connected through peptide bonds. MCs produce by some cyanobacteria genera, including *Microcystis spp.*, *Anabaena spp.* and *Planktothrix spp.* (De Figueiredo et al., 2004). The chemical structure of MC (Figure-1-2) is a monocyclic heptapeptide and the varieties of MCs result from the substitution of different amino acids at particular positions within the ring. MC heptapeptide is composed of *D*-alanine at position 1, two variable L-amino acids at positions 2 and 4, g-linked D-glutamic acid at position 6, D-methylaspartic acid

(MeAsp) at position 3, (2*S*,3*S*,8*S*,9*S*)3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) at position 5 and *N*-methyl dehydroalanine (MDha) at position 7 (Carmichael, 1992). Because of the substitution of different amino acids, there are more than 90 congeners of MCs described (Niedermeyer et al., 2014) and each congener has some differences in its physiochemistry and toxicity. Among the seven amino acids, Adda amino acid is frequently linked to the toxicity of the MC molecule (Dawson, 1998). The most common congener of MC is microcystin-LR (MC-LR) which has leucine (L) and arginine (R) at the variable positions 2 and 4, respectively (Gupta et al., 2003). Other MC congeners frequently detected in surface water are microcystin-RR (MC-RR), and microcystin-YR (MC-YR) (Kuiper-Goodman et al., 1999).



MC variant	X	Z	M. W. (da)
MC-LA	Leu	Ala	909
MC-LL	Leu	Leu	951
MC-LF	Leu	Phe	985
MC-LR	Leu	Arg	994
MC-LY	Leu	Tyr	1001
MC-YL	Tyr	Leu	1001
MC-LW	Leu	Trp	1024
MC-FR	Phe	Arg	1028
MC-RR	Arg	Arg	1037
MC-YR	Tyr	Arg	1044

Figure 1-2: Chemical structure and molecular mass of microcystins (MCs). (A) The generic structure of the MCs. X and Z in positions two and four are highly variable L-amino acids that determine the suffix in the nomenclature of MCs. (B) Microcystin-LR (MC-LR), with the amino acid leucine (L) and arginine (R) in the variable positions two (X) and four(Z), respectively. (C) The molecular masses are of some of the most frequent MCs. This figure caption used by Chen et al. (2016).

1.4. Microcystin physiochemistry and decomposition

The physiochemistry properties of MCs are crucial, as they control the environmental fate and exposure of MCs in organisms. The MCs have many physicochemical properties, such as the aqueous solubility of the solid (SS) (Codd et al., 2005; Pérez and Aga, 2005; Harada, 2004; Liang et al., 2011) and the *n*-octanol-water partition coefficient (K_{OW}) (Skrajnowska et al., 2013). These physiochemical characteristics are important because they influence the environmental and biochemical properties of MCs (Dai et al., 1998).

MCs have a molecular weight between 900 and 1100 Daltons, are highly stable and readily soluble in water (Rivasseau et al., 1998; Sivonen and Jones, 1999) at concentrations > 1g/L (Rivasseau et al., 1998), which can lead to high aqueous concentrations. For example, in river water, if the MCs rise to 5 μg/L, only 10% could be adsorbed on particles and 7% on the sandy sediment after three days (Rivasseau et al., 1998).

The potential for MCs to accumulate in fish tissue is not likely to be related with MC-K_{OW}. K_{OW} is the concentration of a substance in the octanol phase divided by the concentration of the water phase and it is well recognized as a critical property of toxicants (Cash, 1999; Hansen et al., 1999). The K_{OW} of persistent neutral organic compounds indicates the tendency of a compound to concentrate in the lipids of organisms or the organic carbon components of sediments and soils (Karickhoff et al., 1979; Mackay, 1982). MC-K_{OW} is normally lower than 1, which suggests that MC should be excreted relatively easily from the body. However, numerous studies have documented the presence of MC contamination in wild aquatic animals, such as fish (Magalhaes et al., 2001; Mohamed et al., 2003), mussels (Watanabe et al., 1997) and snails (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003). Chen et al. (2005) studied the tissue distributions and seasonal dynamics of the hepatotoxic MC-LR and MC-RR in a freshwater snail (*Bellamya aeruginosa*) from a large shallow, eutrophic lake of subtropical China during June–November 2003. Their results showed that MC accumulation was highest in the hepatopancreas (mean 4.14 and range 1.06–7.42), followed by the digestive tract (mean 1.69 and range 0.8–4.54) and gonad (mean 0.715 and range 0–2.62), whereas the feet had the lowest MC (mean 0.01 and range 0–0.06). Chen et al. (2005) used HPLC with a qualitative analysis using a Finnigan LC-MS system

to analyse MC concentrations. These results showed that the high accumulation of MCs in the different tissue is because MC is binding to the tissue and is accumulated in them. The reduction in surface water concentration of MC after a bloom event is because of a combination of physical, chemical and biological decomposition, and dilution. MCs can persist for months or up to 1 year in natural waters and the dark. Also, MCs can resist the traditional water treatment processes (Harada et al., 1996; Kuiper-Goodman et al., 1999). In addition, MCs can tolerate chemical hydrolysis, oxidation or heating because their cyclic structure enhances their stability (De la Cruz et al., 2011).

The thermal decomposition factor could be considered the less factor to decompose MCs in comparison to chemical decomposition in the environment. According to Harada et al. (1996), the thermal decomposition of MC-LR at pH 9 and 21-30 °C reached the half-life after three weeks and at pH 1 and 40 °C reached the half-life after ten weeks. Their results mimic the real environmental conditions in summer (Harada et al., 1996; WHO, 1999). Whereas, Tsuji et al. (1997) demonstrated that the chemical degradation such as chlorination decomposition was very effective against the toxins. For example, Tsuji et al. (1997) showed that sodium hypochlorite at a concentration of 2.8 mg/L chlorine for 30 min., easily decomposed MC-LR and effectively 99% removed from the water. Tsuji et al. (1997) results confirmed the findings of Nicholson et al. (1994) regarding the effectiveness of chlorination to decompose MC-LR.

In the environment, photolysis can contribute to MC-LR decomposition. In full sunlight under field conditions, particularly when water-soluble pigments are present, the half-life of MCs were approximately ten days (Tsuji et al., 1994). The stability of MCs under photolysis at UV wavelengths near the maximum absorption of the toxins was also dependent on light (Tsuji et al., 1995). Their results showed that MCs easily decomposed by UV light and the half-life of MC-LR by 147 W/cm^2 UV irradiation was ten min. and the MC-LR completely decomposed by 2550 147 W/cm^2 UV after 10 min.

Biological decomposition can be effective at the removal of MCs from the environment (Sivonen, 1990; Lehtimaki et al., 1997; Cousins et al., 1996; Rapala et al., 1997; Orr and Jones, 1998). Interestingly, some bacterial proteases (Rapala et al., 2005) and many aquatic plants and animals can break down MCs (Zurawell et al., 2005). However, in some cases, the toxins can persist for months or even years once released

into cooler and dark natural water bodies (Rapala et al., 2005). MCs are normally present inside cyanobacterial cells and enter the surrounding water after cell lysis. According to Ji et al. (2008), the main route of detoxification of MC-LR is likely through biodegradation (Lahti et al., 1997; Miller and Fallow field, 2001; Ishii et al., 2004). Remarkably, some kinds of bacteria, like *Pseudomonas* spp., which are isolated from the surface water of lakes, rivers and dams, can decrease amounts of MCs. Takenaka and Watanabe (1997) isolated four kinds of bacteria classed in the genera of *Pseudomonas*, *Citrobacter*, *Enterobacter* and *Klebsiella* from the surface water of a Japanese lake where a heavy water bloom occurs every year and tested the bacterial degradation ability of MC-LR. They found that only a bacterium identified as *Pseudomonas aeruginosa* degraded MC-LR. A study by Ji et al. (2009) demonstrated the total of MC-LR and MC-RR, extracellular MC-LR and MC-RR at the beginning of a bloom event and were respectively 0.23–8.93, 0.14–4.73, 0.12–1.15, 0.02–0.17 μg/L by using HPLC for measuring the samples that were taken from Lake Taihu. The average removal efficiencies of these congeners were 67.0%, 40.5%, 40.0% and 66.0%, respectively. Meanwhile, Edwards et al. (2008) reported that degradation of MC-LR and other congeners differed significantly based on location. For example, the half-life (1/2 day) of MC-LR in Lochs Rescobie and Balgavies was four days, Loch Leven, five days; Forfar Loch 9 days and Rivers Carron and Cowie 13 and 14 days, respectively. Edwards et al. (2008) concluded that the degradation of MCs appears to be extensive and not reliant on prior exposure and MC amounts had a significant effect on the degradation by the natural microbial population in the different water samples as seen by delayed degradation in Forfar Loch and enhanced degradation in Loch Rescobie.

1.5. Microcystin-LR

Amongst the microcystin (MCs) toxin types, MC-LR is generally accepted as the most toxic and the most investigated congener (Abdel-Rahman et al., 1993; Codd et al., 2005; Luckas et al., 2005). MCs could represent a tumour-promoting activity among the different kinds of cyanotoxins, particularly MC-LR (Falconer, 1991). When MCs contaminate drinking water at low nanomolar concentrations, this is considered a risk factor for cancer (Falconer, 2007). The accidental death of 100 patients out of 131 at a Brazilian dialysis centre in Caruaru in 1996 was attributed to the use of water polluted with MCs for hemodialysis (Azevedo et al., 2002; Jochimsen et al., 1998; Pouria et al., 1998; Yuan et al., 1996). Also, MC-LR has been implicated in the majority of the incidents of human toxicity that involve MCs (Falconer, 1991). As a result, the

World Health Organization (WHO) has proposed a provisional maximum guideline threshold concentration of 1.0 µg/L for microcystin-LR in drinking water (WHO guidelines, 2003). It is difficult to predict the toxicity of cyanobacterial blooms under natural conditions because this differs among blooms of same species and likely varies with time within given species (Table 1-1). Furthermore, several studies have reported that the toxicity (Codd et al., 1989) and the toxin content of the cells (Watanabe et al., 1997) were maximal at the late exponential growth phase.

Table 1-1: Examples of different concentrations of MCs congeners in various places around the world, during the bloom event. *Example from Transvaal Dams which is Schoonspruit 1989, **Lake Suwa example from year 29/09/1994.

Location	MCs Congeners	MCs concentration µg/g DW	Analysis method	Reference
South Africa*	MC-YR,	10	HPLC	Scott, 1991
	MC -LR,	180		
	MC-FR,	90		
	MC-YA,	30		
	MC-LA,	240		
	MC-LAba	80		
Japan**	MC-LR	89.9	HPLC	Park *et al.*, 1993
	MC-RR	136		
	MC-YR	Not detected		

When MCs release into the aquatic environment, they will absorb by organisms and they will rapidly accumulate in the liver (Fischer et al., 2000 c). Then, MCs will bind to protein phosphatase 2A (PP2A) (Dawson, 1998; Gupta et al., 2003; Kuiper-Goodman et al., 1999; MacKintosh et al., 1990). The binding to PP2A can inhibit it, leading to hyperphosphorylation in the liver, which leads to cell necrosis, massive haemorrhage and death. For example, the lethal dose 50 (LD50) of MC-LR after intraperitoneal (i.p.) injection in mice was in the range from 25 to 150 μg/kg (Kuiper-Goodman et al., 1999). This range could be different according to the MC congeners, but MC-LR is usually used as a reference.

MCs are considered possible tumour promoters (Falconer, 1991). According to Liu and Sun (2015), MCs can induce different cellular toxic effects, such as enhance cancer cell invasion, cause oxidative damage and alter the stabilisation of cytoskeleton that will lead to cellular apoptosis. The cellular effects that induced by MC are not enough to understand the MC toxicity mechanism. Consequently, a better understanding of the mechanism of MC toxicity will be revealed, after identifying the critical molecules or pathways that involved in MC-induced toxicity. Moreover, the cellular effects that are accredited to the role of several proteins and enzymes. For example, PP2A is well studied in MC toxicity, as it is considered a crucial regulator in MC-induced molecular network.

The PP2A with PP1 together belong to the PPP family that is contributing most of the serine/threonine phosphatase activity in cells. The well-knowing the mechanism of toxicity of MCs is that MC-LR is a potent inhibitor of PP2A/PP1. The inhibition of PP2A/PP1 causes hyperphosphorylation that can relate to MC-LR toxicity and tumour promotion activity. Moreover, the role of PP2A in MC-LR induced toxicity is being well studied, as MC-LR has higher affinity and inhibitory ability to PP2A than PP1. PP2A plays key roles in the regulation of a wide range of cellular processes because it is considered an important serine/threonine phosphatase. The main forms of PP2A are dimers of catalytic (C) and scaffolding (A) subunits and trimers with an additional variable regulatory B subunit and MC-LR bind to the active-site pocket of the catalytic subunit of PP2A (Strack et al., 2004). The balance between protein de-phosphorylation and protein phosphorylation is a vital mechanism, which regulates signal transduction in eukaryotic cells. This balance is a dynamic change that is nearly involved in all the processes from embryonic development to mature adults. Thus, the reduced PP2A activity by MC-LR exposure can affect a series of key cellular effects, such as gene

expression, cell cycle, cell proliferation, division and signal transduction (Sun et al., 2014).

When MCs are released in the water during the decomposition of cyanobacteria blooms, a wide range of aquatic organisms, especially fish, are exposed directly to the dissolved toxins. According to Ishida et al. (1999), Rohrlack et al. (2003) and Yamaki et al. (2005) exposure larval fish to *Microcystis* blooms in the environment is a complex issue because the larval fish are not exposed to only MC-LR, but to *Microcystis* cells and numerous other substances in the aquatic environment. Furthermore, *Microcystis* can produce several toxic peptides classified as aeruginosins (Ishida et al., 1999), micropeptins (Yamaki et al., 2005) and microviridins (Rohrlack et al., 2003), which may contribute to toxicity (Harada, 2004; Smith et al., 2008). Moreover, *Microcystis* cell walls contain lipopolysaccharides that can contribute to toxicity (Jaja-Chimedza et al., 2012). Many studies have shown that the early life stages of zebrafish, when exposed to *Microcystis* and MC-LR together, were more affected than by exposure to MC-LR alone (Oberemm et al., 1997; Oberemm et al., 1999; Best et al., 2001; Palikova et al., 2007).

1.6. Microcystin (MC) toxin exposure routes

The exposure routes of MCs in the real environment may occur through different pathways. The two main exposure ways are the oral uptake of toxin-containing cells and via epithelium surfaces by immersion in water containing the dissolved toxin or both routes combined (Malbrouck and Kestemont 2006). In many previous studies, they did not use the environmentally relevant exposure scenarios. However, the method that used in the most of previous studies was the aqueous exposure route of MC as an acute exposure during the early life stages of fish (Best et al., 2002; Best et al., 2003; Oberemm et al., 1997; Oberemm et al., 1999; Wiegand et al., 1999). Additionally, few studies have conducted chronic aqueous exposure with adult fish (Adamovsky et al., 2007; Carbis et al., 1996 a, b; Li et al., 2005; Mares et al., 2009; Qiao et al., 2013). Besides that, another type of acute exposure to MC found in the literature is the microinjection (Huynh-Delerme et al., 2005; Jacquet et al., 2004; Wang et al., 2005). The microinjection exposure aims to imitate uptake of toxin from the surrounding water by the embryo or by transferring MC from females to eggs. On the other hand, the occurrence of maternal transfer has not developed for MC because this kind of exposure is somewhat hypothetic or an imaginary route (Malbrouck and Kestemont, 2006). Other studies have investigated the exposure of MC through oral gavage, the ingestion route (Fischer et al., 2000c;

Tencalla and Dietrich, 1997) and intraperitoneal injection in their exposure methods (Fournie and Courtney, 2002; Malbrouck et al., 2003). Other researchers included either *Microcystis* cells or MC-LR into fish feed (Dong et al., 2009; El-Ghazali et al., 2010; Li et al., 2004; Li et al., 2005; Soares et al., 2004; Deng et al., 2010; Acun et al., 2012), which is similar to the natural feeding in the real environment.

1.7. Ecotoxicology of *M. aeruginosa* / MC-LR in fish

1.7.1. Ecotoxicological effects of *M. aeruginosa* / MC-LR in fertility parameters and gonad histopathology

M. aeruginosa and MC-LR have negative effects on fish fertility and gonads histopathology. The gonad is considered the second crucial target for MCs (Chen et al., 2005; Chen et al., 2011; Chen et al., 2013). Numerous studies have shown that MCs can accumulate in gonads in the range 0–2.62 µg/g DW after the liver (Chen et al., 2005; Chen et al., 2011; Chen et al., 2013; Lance et al., 2010; Papadimitriou et al., 2009). MC accumulation can cause lesions in testes and ovaries as Trinchet et al. (2011) demonstrated in their study through testing the effects of MC-LR on reproductive function in medaka fish (*Oryzias latipes*). According to Trinchet et al. (2011), the changes that happened in ovaries included a decrease of vitellus storage, lysis of the gonadosomatic tissue and disruption of the relationships between the follicular cells and the oocytes, whereas, in the males, spermatogenesis seemed to be disrupted (Trinchet et al., 2011).

MC-LR can cause morphology changing and mortality on fish. Malbrouck and Kestemont (2006) reported in their review several studies which illustrated the effects of MCs generally and MC-LR, particularly on fish, including zebrafish. For example, a study by Wang et al. (2005) illustrated that depending on the dose and the time of exposure to MC-LR the findings showed that mortality and various abnormalities in zebrafish such as trunk and tail curving, bent or twisting tails, edema in pericardial sac and hatching gland occurred. Qiao et al. (2013) reported that MC-LR had negative effects on the reproductive system of male and female zebrafish in different aspects, but some of their results were insignificant and they contend that the female is more vulnerable than males to MC-LR.

The mechanism of reproductive toxicity of MC-LR has been investigated. Qiao et al. (2013) investigated this in zebrafish by sub-chronic aqueous exposure of adult

zebrafish to MC-LR (1, 5 and 20 μg/L) for 30 days. They showed that the number of eggs spawned decreased but was not significant in all the treatment groups. The hatchability declined significantly in the group exposed to 20 μg/L of MC-LR and the concentration of testosterone decreased, but not significantly. The gap of information here is that Qiao et al. (2013) did not use the crude aqueous extracts of cyanobacteria with MC-LR to see the different effects that would have. Also, if they used the feeding exposure, what would be the results on the reproductive system for each gender? Another study by Chen et al. (2013) showed that MC-LR has negative effects on the reproductive system by investigating the interactions between cytoskeleton disruption and mitochondria dysfunction of rat testes *in vivo* leading to reproductive toxicity.

Earlier studies investigated the reproductive toxicity and histopathological changes in rats resulting from exposure to MCs. The previous results confirmed that MCs could accumulate in testis and caused toxic effects on the reproductive system (Li et al., 2008; Liu et al., 2010; Xiong et al., 2010; Zhao et al., 2012; Li and Han, 2012). MCs lead to morphological damages to gonad (Li et al., 2008; Li and Han, 2012; Ding et al., 2006) and can cause a significant decline of sperm quality (Li et al., 2008; Ding et al., 2006; Chen et al., 2011). Additionally, MCs can affect some serum hormones, including testosterone, follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels and leading to decrease in their levels (Li et al., 2008). Few studies reported that MCs induced ultrastructure damage of testis (Liu et al., 2010; Zhao et al., 2012). The results of Chen et al. (2013) showed that after intraperitoneal injection of MC-LR on male rat for 50 days with concentrations of 1 and 10 mg/kg of body weight per day, the testes index (calculated by dividing the testes weight by the body weight) decreased significantly after 12 hours of the last intraperitoneal injection. Additionally, in the group that exposure to dose 10 mg/kg, the hormone levels of testis changed significantly. The histopathological results showed enlarging the space between the seminiferous tubules, shrinkage of cytoplasm, cell membrane splotching, swollen mitochondria and deformed nucleus. Another question to be built on this study would be to ask what will be the effect on the testes if they will adopt the feeding exposure of lyophilized cells of *M. aeruginosa* or MC-LR to see the different impact on testis? Or are the same morphological changes will happen with adult zebrafish after adopting the dietary exposure?

Previous studies investigated the reproductive toxicity and histopathological changes of *Microcystis* cell extracts on mice. A study by Ding et al. (2006) examined the toxic effects of *Microcystis* cell extracts containing MCs on the reproductive system of male mice. Ding et al. (2006) exposed male mice intraperitoneally to doses of 3.33 or 6.67 mg MC/kg body weight for 14 days. There was a decline in the body weight mean, the absolute weight of testes and epididymitis, the mature sperm quality and the motility and viability of the sperm. Furthermore, histopathological tests showed damage between the seminiferous tubules in testis. Conversely, the relative weight of the testes rose compared to the control and no significant effects were found the concentration and abnormality of the sperm. Ding et al. (2006) exposed i.p. male mice to 33.3 mg MCs/kg and showed that the acute toxicity test of body weight was low, which disagreed with the previous studies (Dawson, 1998; Fawell et al., 1999). Because Dawson (1998) and Fawell et al. (1999) also showed higher body weight with the same dose.

Other studies investigated the reproductive toxicity and histopathological changes of MC-LR on female mammals, such as rat and mice. Wu et al. (2014) investigated the effects of MC-LR on the reproductive toxicity of female rats and mice. Wu et al. (2014) used two different doses of MC-LR with two different organisms, the first one was the same concentrations that were used in (Dawson, 1998) through treated the female Sprague-Dawley rat groups with i.p. lethal dose 50 (LD50) of MC-LR of 200 µg/kg for six days to determine whether MC-LR could enter the ovary. Secondly, female mice were treated with a different dose of MC-LR (0, 5 and 20 µg/kg) by i.p. injection for 28 days to investigate the reproductive toxicity. The results of Wu et al. (2014) confirmed their hypothesis that there were significant effects such as the relative ovary weight decline in the higher dose 20 µg/kg and fall of progesterone level, but no significant effects of LH or FSH.

The reproductive toxicity and histopathological changes of MCs and *Microcystis* on male rabbit were investigated. Liu et al. (2010) used a male rabbit to study the effect of MC through i.p. injection exposure with doses 12.5 and 50 µg/kg MC. Their results confirmed the finding of previous studies that examine the effects of MCs and *Microcystis* on testes in different organisms. Liu et al. (2010) examined the ultrastructure and biochemical index in rabbit testis. With a dose of 50 µg/kg, all the animals died after 3 hours exposure. In the treatment of 12.5 µg/kg, changes happened at 1, 3 and 12 hours on distension of

mitochondria, endoplasmic reticulum and Golgi apparatus and the intercellular junction became wider.

Microcystis cell extracts contain other types of substances, which may increase its toxicity, such as lipopolysaccharides (LPS). Oberemm et al. (1997, 1999) showed that the aqueous crude extracts of cyanobacteria to be more obvious and more significant than MCs. Ding et al. (2006) also reported that the effects of MCs and the crude aqueous extract of cyanobacteria inhibit the embryonic development of amphibians and fish especially zebrafish. Because the ingredients of the crude extracts could increase the uptake rate of toxins and synergistic actions of toxins and unknown substances existing in freshwater, which means increasing the toxicity of cyanobacteria.

The sub-lethal concentrations of MC-LR could cause biochemical responses in various organs of zebrafish. A study by Pavagadhi et al. (2012) contended that the sub-lethal concentrations of MC-LR and MC-RR to which zebrafish (*Danio rerio*) were exposed under balneation conditions i.e., aquatic organisms being bathed in the water containing the trace levels of dissolved toxins. Balneation condition helps to make a realistic evaluation of MC toxicity through chronic and subchronic exposure routes. Their results depended on the assessment of oxygen-mediated toxicity in the tissue of liver, gills, intestine and brain of zebrafish. Most of the enzymes followed a bell-shaped curve, with a rapid increase in activity at some concentration. Some of the enzymes have shown an adaptive response after the first-time exposure, whereas enzyme activity has increased in some tissues. This study does not discount the previous studies but builds on them to include the effects of MCs and *Microcystis*.

So far, very few studies have used fish to investigate the effects of MCs / *M. aeruginosa* on fertility and histopathology. Also, no previous study had used the dietary exposure of MCs / *M. aeruginosa* to investigate the effects on the reproductive system and the histopathology changes.

1.7.2. Ecotoxicological effects of *M. aeruginosa* / MC-LR in early life stages in fish

Early life stages of fish are more affected generally by toxins in compared to adults or juveniles. MC-LR affects the critical development stages of embryos and larvae (Buryskova et al., 2006). Recently, there were numerous studies, which examined the effects of different congeners of MCs on the embryonic development and larval growth of aquatic organisms such as Oberemm et al. (1997 &1999); Wiegand et al. (1999); Liu et al. (2002); Wang et al. (2005); Wright et al. (2006); Malbrouck and Kestemont (2006); Palikova et al. (2007). Previous studies reported that pure MC-LR could cause severe damage in embryos through using a microinjection technology and these studies have been thought that the external exposure to MCs is a limitation to fish embryonic development (Jacquet et al., 2004; Wang et al., 2005). Conversely, some experiments which used cyanobacterial crude extracts to observe the prominent inhibition effects of MCs on the development of embryos, which were incubated in the toxicant solution (Oberemm et al., 1997, 1999; Palikova et al., 2007; Buryskova et al., 2006). Compared to the effect of pure MC-LR, the effects of crude extracts of cyanobacteria were much more obvious, possibly due to a high uptake rate of the toxin.

MCs could cause low hatching rate and high malformation (Oberemm *et al.*, 1997, 1999; Wiegand et al., 1999; Liu et al., 2002; Jacquet et al., 2004; Wang et al., 2005). MCs are believed to affect larval or juvenile development with high mortality, growth inhibition and histopathological changes (Oberemm et al., 1997; Palikova et al., 2007). Oberemm et al. (1997, 1999) investigated exposing different congeners of MCs /LR, RR and YR, and the crude aqueous extracts of cyanobacteria on fish such as the embryos of zebrafish and the stone loach. The results showed that MC-LR in doses 0.5 and 5 μg/L did not reveal significant implications during the early embryonic development, but in higher doses of MC-LR (50 μg/L) significantly reduce in the survival rate, weight and total body length. However, the effect of the crude extract of cyanobacteria was noted to be more obvious and significant. Their results indicated that the effects of pure MC-LR compared to the effects of water crude extracts were much less evident, possibly due to a very low rate of uptake of the toxic environmental concentrations of pure MCs are not acutely toxic to the fish's eggs. However, Wang et al. (2005) question the reliability of the results of Oberemm et al. (1997) and Oberemm et al. (1999) when they were studying the inhibition of embryonic development by MC-LR in zebrafish. Wang et al. (2005) found that MC-LR caused the death of zebrafish embryos in a manner equal with the time and dosage and conducted that the toxicity responses of embryos

could be a useful bioassay system for detecting MC-LR. Malbrouck and Kestemont (2006) demonstrated in their review many studies that studied the effects of MCs in different life stages in different histopathological aspects in different aquatic organisms, particularly zebrafish. For example, Wiegand et al. (1999) examined the uptake of MC-LR by different early life stages of zebrafish through using ^{14}C-radiolabeled MC-LR. A detectable uptake of MC was observed from the first day of embryonic development up to 5 days old larvae. On average, absorption of 0.5 ng MC per egg was calculated over the entire exposure time.

1.8. Effects of MC-LR / *M. aeruginosa* on zebrafish gene expression profile and enzyme activity

The effects of *Microcystis* sp. and MC-LR on gene expression and enzyme activities have been investigated in various species of fish. The level of enzyme activity depends on the translation of mRNA and how fast the enzyme has been destroyed against to different toxic substances. Previous studies have considered different routes of exposure to either MC-LR or *M. aeruginosa* and testing the effects of MC-LR or *M. aeruginosa* on different stages of development in different organisms, especially fish. In the context of toxicity, of particular interest are genes involved in reproduction vitellogenin (*VTG1*); oxidative stress catalase (*CAT*), superoxide dismutase 1 (*SOD1*) and glutathione peroxidase (*GPx*); liver protein phosphatase (*PPP1ca*); and biotransformation genes i.e., cytochrome P450 (*CYP1A1*) and glutathione-S-transferase 1 (*GST1*).

1.8.1. The effects of *M. aeruginosa* / MC-LR on vitellogenin (*VTG1*) gene expression

VTG has widely considered as a vital indicator of reproductive status in fish. The *VTG* gene expression is under estrogenic regulation and generally expressed in female fish. Though, *VTG* can be induced in male fish by xenoestrogens through binding to hepatic estrogenic receptors (Pakdel et al., 1991; Sumpter and Jobling, 1995). The genome of zebrafish contains seven *VTG* genes (*VTG1–7*) and the level of *VTG1* mRNA is 100–1000 times higher than other *VTG* genes mRNAs (Wang et al., 2005). *VTG1* gene expression level is an important indicator to detect the environmental estrogens (Marin and Matozzo, 2004). *VTGs* are synthesised in the liver, then secreted into the blood circulation and incorporated into oocytes as yolk (Aravindakshan et al., 2004). Changes in vitellogenin gene (*VTG1*) expression have been investigated and evaluated in few studies in the context of the toxicity of *M. aeruginosa* and MC-LR.

So far, there were three studies regarding *Microcystis* / MC-LR exposure with zebrafish in different life stages i.e., Rogers et al. (2011); Qiao et al. (2013) and Zhao et al. (2015).

A. Larval zebrafish

Changes in vitellogenin gene (*VTG1*) expression in zebrafish larvae has been investigated and evaluated in one study in the context of the toxicity of *M. aeruginosa* / MC-LR. A study by Rogers et al. (2011) found induction of *VTG*1 gene when they exposed zebrafish to *M. aeruginosa*, but not to MC-LR. Their results showed that the *VTG*1 gene was highly upregulated from 19.2-fold to >100-fold, upon aqueous exposure of larval zebrafish to *M. aeruginosa* (containing 4.5 µg/L MC-LR) at doses 100 and 1000 µg/L for 96hpf which is consistent with the estrogenic response, but no *VTG1* expression when the larvae were exposed to MC-LR in the same doses. So, these results reflect that MC-LR does not have an estrogenic effect, but, high induction of *VTG1* was found with *M. aeruginosa* (containing 4.5 µg/L MC-LR) at the same doses, which means that the other substances that *M. aeruginosa* contains may cause the estrogenic response. There are not any other studies to make a comparison with Rogers et al. (2011) paper. However, the level of *VTG1* induction is consisted with the exposure to 17 β-estradiol (E2), besides that the zebrafish larvae is considered a tool to assist whether if there is an estrogenic effect or not for some substances that can cause *VTG1* induction or reduction.

B. Adult zebrafish

Changes in vitellogenin gene (*VTG1*) expression in adult zebrafish has been investigated and evaluated in two studies in the context of the toxicity of *M. aeruginosa* / MC-LR. Qiao *et al*. (2013) results showed that down-regulation of *VTG1* in the liver of adult zebrafish after chronic aqueous exposure for 30 days of MC-LR at these doses 5, 20 µg/L, as the female's liver showed decreasing from 2000 fold in control down to ~ 900 fold in the treatment groups and for the male's liver showed decreasing from 0.6 fold in the control down to ~ 0.4 fold in the treatment groups. However, a study by Zhao et al. (2015) found that after exposing the fish for 21 days to MC-LR in doses 10 and 50µg/L that *VTG1* expression in the liver induced up to 4-fold change, but down-regulated in the liver when zebrafish were exposed to 50 µg/L. The similarity between these two studies that they used (E2) levels in the plasma to compare the *VTG1* results.

VTG1 gene is usually used as a biomarker for estrogenic compounds. The previous three studies explored the estrogenic effect of either MC-LR / *M. aeruginosa* together on zebrafish larvae or MC-LR on adult fish and they reached to

different conclusions. These differences may be due to different developmental stages of zebrafish, different MC-LR / *M. aeruginosa* exposure concentration, different exposure duration and different tissue samples that used in each experiment. However, still, there are some gaps in understanding that need to fill. If a comparison is made between Zhao et al. (2015) and Qiao *et al*. (2013), it can see that the aqueous exposure of MC-LR or *M. aeruginosa* in different lower and higher doses made an induction for *VTG1*, so what about the dietary exposure for either MC-LR or *M. aeruginosa* are the results will be confirming or it will be different?

1.8.2. The effects of *M. aeruginosa* / MC-LR on oxidative stress enzyme activity

Oxidative stress can be considered a toxicological consequence of the exposure to MCs in different organisms. Inhibition PP1 and PP2A by MC is the classical toxicity mechanism, which is described in many previous reports such as zooplankton (DeMott and Dhawale, 1995), amphibians (Fischer and Dietrich, 2000a), fish (Fischer and Dietrich, 2000b; Fischer et al., 2000c) and mammals (Chen et al., 2006b).

Oxidative stress is a disturbance in the balance of prooxidant and antioxidant, which can lead to potential molecular damage (Halliwell and Gutteridge, 2007). Previous evidence indicates that MCs could induce oxidative stress and/or alter the antioxidant system in various aquatic species and organs. MC uptake has associated with the production of reactive oxygen species ROS (Ding et al., 2000, 2001; Li et al., 2003) that can lead to an increase in lipid peroxidation (Pinho et al., 2005; Jos et al., 2005; Prieto et al., 2007), DNA damage (Zegura et al., 2003, 2008; Votto et al., 2007), DNA protein crosslink (Leão et al., 2008), mitochondrial damage (Ding and Ong, 2003) and alteration of the antioxidant defence system (Vinagre et al., 2003; Pinho et al., 2005; Cazenave et al., 2006 a, b; Prieto et al., 2007; Amado et al., 2009b).

The bloom of some cyanobacteria genera i.e., *Microcystis* and *Anabaena* can simply alter oxidative stress by generating hyperoxia/anoxia cycles through photosynthetic and respiratory processes (Seki et al., 1980). Rosa et al. (2005) suggested that a cycle of high /low oxygen levels in the water column could explain the extremely high levels of lipid peroxidation in the estuarine worm *Laeonereis acuta* (Nereididae) that was collected under a bloom event dominated by cyanobacteria genera such as *Anabaena.* Moreover, the previous in vitro studies showed that the ROS production is also a metabolic response to MCs exposure and not only an effect that is corresponding to

ischemia/reperfusion process. Ding and Ong (2003) elevated ROS levels in rat hepatocytes after just 5 min. exposure to 1 μM MC-LR. Also, high levels were reported of ROS generation for fish hepatocytes and lymphocytes after 30 and 90 min of MC exposure respectively (Li et al., 2003; Zhang et al., 2007).

MCs could induce oxidative stress and could change the antioxidant system, in a variety of terrestrial and aquatic species. Increasing the evidence that suggests the excessive ROS production can play an important role in the toxic mechanism of MCs. When antioxidant defence system does not counter the ROS production when it is getting high, cellular oxidative stress occurs. The antioxidant system can counter by the antioxidant enzymes, i.e., CAT, GPx and SOD. SOD catalyses the conversion of superoxide to hydrogen peroxide and CAT and GPx reduce hydrogen peroxide to H_2O (Amado et al., 2010). Previous studies focused on the enzyme activity for the oxidative stress on mammalian species such as rat and rabbit and few studies have been studying the aquatic animals, for example, Medaka fish and zebrafish. So far, just four studies have focused on zebrafish (different life stages) and the enzyme activity of the oxidative stress, not the gene expression.

A. Embryos and larval zebrafish

MCs can affect oxidative stress in early life stages in zebrafish. Wiegand et al. (1999) investigated the uptake of MC-LR in the different life stages of zebrafish through using ^{14}C - labeled MC-LR from first embryonic development up to 5 days old larvae. They immersed the embryos in REKO medium which contained 0.1, 0.5, 1, 2 and 5 μg MC-LR/L over ontogenetic development and after hatch (3 and 5 days). Some of their results showed that GPx enzyme activity increased in 0.5 μg/L. Similarly, another study by Cazenave et al. (2006a), they used zebrafish embryos which were immersed in REKO medium with either 25 μg MC-RR/L or 25 μg MC-LF/L, but in just 24 hours. Some of their endpoints were to determine the changes in CAT, GPx and GR enzyme activities. The results showed that there were no changes in the enzyme activity of CAT, GPx and GR. In order to compare between these two studies that the enzyme activity for some of the oxidative stress enzymes induced in low doses of MC-LR and the evidence that Cazenave et al. (2006b) showed that there were no changes in the activity of oxidative stress enzymes during the acute 24h exposure to other MC congeners. Still, there is a gap to fill regarding MC-LR and antioxidant-related genes in larval zebrafish.

B. Adult zebrafish

MCs can affect oxidative stress in adult zebrafish. Liu et al. (2014) exposed aqueously adult zebrafish to three different concentrations of MC-LR (1, 5 and 20 µg/L) for 30 days and after MC-LR exposure the fertilized eggs were collected and the following F1 generation was reared in water containing no MC-LR until 60 days post fertilization (dpf). The activities of some antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), significantly dropped in the groups that treated with 5 and 20 µg/L MC-LR. However, a study by Hou et al. (2014) injected (i.p.) female zebrafish to acute toxic exposure of MC-LR at concentrations of 50 and 200 µg/kg body weight MC-LR. Within 12 hours post injection, the levels of antioxidant enzymes CAT, SOD and GPx increased, indicating the occurrence of oxidative stress. To compare between these two studies, it seems that the higher doses i.p. injection for a short time induced the oxidative stress, whereas the opposite happened with the chronic aqueous exposure, we can say that even the dose, the way of exposure and the time are crucial, but the oxidative stress could be induced sooner than later. The question is what the results could be when the dietary exposure is adopted for both MC-LR and *Microcystis*?

1.8.3. The effects of *M. aeruginosa* / MC-LR on biotransformation gene expression and enzyme activity

Many different toxins that come from eaten, breathed or created in our bodies are broken down by the Cytochrome P450 (CYP450) family of enzymes in what is known as phase one of detoxification or biotransformation. Most of the biotransformation takes place in the liver, which is considered a main organ for detoxification. CYPs450 belong to a superfamily of heme-monooxygenases that catalyze steroidal hormones and oxidation of endobiotic lipids, besides many xenobiotic chemicals, including drugs, carcinogens and environmental contaminants and breaks them into smaller substances (Singh et al., 2011; Nelson, 2003). CYP1, CYP2 and CYP3 among the many CYP isoenzymes are three main families of the CYP superfamily, which are involved in biotransformation and metabolism in a wide variety of xenobiotic chemicals (Singh et al., 2011). Also, CYP450 enzymes contain oxygen and iron and by a redox reaction can make a drug more polar. The polar molecules are more hydrophilic and can able to be eliminated through the kidneys. Besides that, the CYP450 family of genes have various polymorphisms, which can either slow down or speed up the rate of the enzyme (Zhao et al., 2015; Amado et al., 2009b).

CYP1A1 is a liver biotransformation gene, which codes for the enzyme of the same name and takes part of the phase one metabolism of some drugs and xenobiotics in the liver. Also, *CYP1A1* involve the activation of aromatic hydrocarbons (PAH) in the intestines, involve the metabolism of 17β-estradiol and the polyunsaturated fats arachidonic acid (Amado et al., 2009b).

GSTs is part of an ongoing series on the genes involved in phase two biotransformation. GST enzymes have eight classes identified: alpha, kappa, mu, omega, pi, sigma, theta and zeta. Phase two enzymes add glutathione to toxins to detoxify them. GSTs are found in the liver, intestines and many other places in the body. In addition, these enzymes are responsible for biotransforming a big number of pesticides, herbicides, carcinogens and chemotherapy drugs. After a toxic substance has been conjugated with glutathione via the GST specific enzyme, it can be excreted from the body via bile or urine. Glutathione is considered the main antioxidant for the body (Hou et al., 2014).

MC effects have been investigated on biotransformation enzyme activities in previous studies. Changes in cytochrome P450 (*CYP1A1*) gene expression have not investigated in the context of the toxicity of *M. aeruginosa* or MC-LR. In addition, glutathione-S-transferase 1 (*GST1*) have not been investigated and evaluated in the context of gene expression, as one study was focused just on the enzyme activity. So far, there are two studies regarding *Microcystis* / MC-LR exposure with adult zebrafish, i.e., Zhao et al. (2015); Hou et al., (2014), as there is no study regarding zebrafish larvae with *M. aeruginosa* / MC-LR and biotransformation related genes. Zhao et al. (2015) exposed aqueously female zebrafish sub-chronically to MC-LR in concentrations 2, 10 and 50 µg/L for 21 days. Their results showed that *CYP19A*, *CYP19B* and *CYP17* have changed after the exposure and corresponded well with the alterations of hormone levels, as the concentrations of E2 and VTG hormones at the 10 µg/L level were increased, but E2, VTG and testosterone concentrations were decreased at 50 µg/L MC-LR. Another study by Hou et al. (2014) exposed acutely female zebrafish to MC-LR by (i.p.) injection of 50 and 200 µg/kg body weight. The results showed that within 12 hours post injection, the levels of antioxidant enzymes CAT, SOD, GPx and biotransformation enzyme GST increased. The limitations on the previous studies are that no information available regarding *CYP1A* and *GST1* genes expression on zebrafish larvae after exposing to either *M. aeruginosa* or MC-LR. Additionally, what will be the results after the sub-lethal dietary exposure of *M. aeruginosa* and MC-LR on adult zebrafish?

1.8.4. The effects of *M. aeruginosa* / MC-LR on protein phosphatase 1 and 2A

Protein phosphatase 1 (PP1) and Protein phosphatase 2A (PP2A) together belong to the PPP family, which is contributing most of the serine/threonine phosphatase activity in cells. Changes in protein phosphatase *PPP1ca* gene expression have not been investigated and evaluated in the context of toxicity of *M. aeruginosa* and MC-LR, as the few previous studies focused just on the enzyme activity in the context of the toxicity of *M. aeruginosa* and/or MC-LR for PP1 and PP2. So far, there were two studies regarding *Microcystis* and/or MC-LR exposure on larval and adult zebrafish and PP1 and PP2 (Wang et al., 2010; Tzima et al., 2017).

MC-LR is considered an effective inhibitor of PP2A/PP1 that could lead to protein hyper-phosphorylation. Protein hyperphosphorylation could explain the MC-LR toxicity and tumour promotion activity (Wang et al., 2010). Also, MC-LR can inhibit PP2A more than PP1, as MC-LR is high affinity (Xing et al., 2006). Consequently, decreasing PP2A activity by MC-LR exposure, could alter some key series cellular effects, such as cell cycle, cell proliferation, division, signal transduction and gene expression (Sun et al., 2014). In the first study by Wang et al. (2010), they adopted adult zebrafish (*Danio rerio*) and aqueously exposed them chronically to MC-LR for 30 days at concentrations (2 and 20 mg/L). Their results showed that no fold change for the expression of *PP2aA* and *PP2aC* with very slightly increasing nearly 1.5fold. However, Tzima et al. (2017), showed that after exposing zebrafish larvae to 50 and 500 µg/L MC-LR for four days revealed 40% reduction of PP2A enzyme activity in comparison to the controls, which may be indicated the early effects of MC-LR.

While various studies have investigated the effects of MC-LR and *M. aeruginosa* in fish, there is still considerable lack of understanding of the molecular mechanisms of reproductive toxicity, oxidative stress gene response, biotransformation gene response and protein phosphatase gene response in larvae and adult zebrafish. Up to now, few articles exist that have addressed the effects of the chronic dietary exposure of MC-LR on *Dorosoma petenense* and medaka without addressing the time relation. Additionally, no information available regarding aqueous exposure of MC-LR / *M. aeruginosa* on time course relation for larval and adult zebrafish. Moreover, the previous studies focused only on the enzyme activities for the different genes. Initial information from proteomics studies provided some new insight into MC-LR toxicity, which indicated that the chronic toxicity of MC-LR is different from

acute toxicity and that oxidative stress might be the main toxic pathway instead of disruption of protein phosphatases (Chen et al., 2016). A critical step is to understand the mechanisms of *Microcystis* / MC toxicity through determining the changes in the gene expression profile in larvae and adult zebrafish and this research area is a priority to investigate.

1.9. The toxicity and the mechanism of MC-LR

During the blooming event of *Microcystis*, MCs are released from the cyanobacterial cells into the water and fish can be exposed during any stage of their life history. In fish, the liver is considered the first target that MC-LR mainly accumulates (Deblois et al., 2011; Papadimitriou et al., 2012). Moreover, MC-LR can also be transported through the blood to other organs such as gonad (Lei et al., 2008; Papadimitriou et al., 2009). Lysis of cells in ovary and disruption of spermatogenesis in testis were observed in medaka fish exposed to 5 μg / L MC-LR, for 30 days exposure (Trinchet et al., 2011). Another study by Adámek et al. (2011) addressed that the cyanotoxicity biomass in a concentration of 200mg/L was significantly affected hatching rates, delayed embryonic development and caused morphological deformities in medaka embryos. In addition, MC-LR toxicity could transmit to offspring (Liu et al., 2014). The findings from Liu et al. (2014) study showed that there was liver damaged and obviously influenced the growth and immune function in the first generation of offspring when a chronic exposure of MC-LR in doses of 5 and 20 μg/L MC-LR were adopted to parents' zebrafish. Furthermore, exposing zebrafish embryos to MC-LR in dose (>100ng/ml) also had adverse effects on early developmental stages of the fish (Pavagadhi et al., 2013b).

The toxicological impacts of MC-LR were studied in different life stages of fish. From the previous works it can be concluded that many studies have focused on toxicological implications of MC-LR on, embryos, larvae and adult fish (El-Ghazali et al., 2010; Li et al., 2011; Zhao et al., 2012). The understanding is limited to whether there are any differences in effect when using *Microcystis* in low doses (aqueous or dietary exposure) for long-term exposure in comparing to MC-LR.

1.10. Gut microbiota

One of the long-term goals of the Human Microbiome Project is to develop effective strategies for manipulating gut microbial communities to promote and sustain the health of human hosts (Peterson et al., 2009). To achieve this goal, it is important to understand the principles governing microbial community assembly and maintenance within the intestine. Zebrafish have been used widely to study vertebrate development, histopathology and physiology. Recently, zebrafish have been established as a model for studying gut microbiota interactions.

Zebrafish normally hatch after two days post fertilization though this depends on the temperature (Villamizar et al., 2012). The zebrafish's mouth opens at day three post-fertilization and the whole gastrointestinal tube is opened at day six post fertilization (Wallace and Pack, 2003). During this time, the yolk sac is consumed and the larvae start to feed on small protozoans such as paramecia. After several weeks post-fertilization, the intestines develop and many bacterial species start to colonize the gastrointestinal tract. The bacterial diversity community in the zebrafish's gut makes zebrafish an excellent model to investigate the essential processes underlying intestinal inflammation and injury (Cheesman and Guillemin, 2007; Yang et al., 2014). Zebrafish have three different gut segments, which are differentiated by morphology and gene expression, i.e., the anterior gut segment (intestinal bulb), midgut and the posterior gut segment (Wallace et al., 2005; Wallace and Pack, 2003). The zebrafish' gut does not have a stomach like mammalian intestine, which has five different intestinal segments, i.e., jejunum, duodenum, ileum, caecum and colon (Figure 1-3).

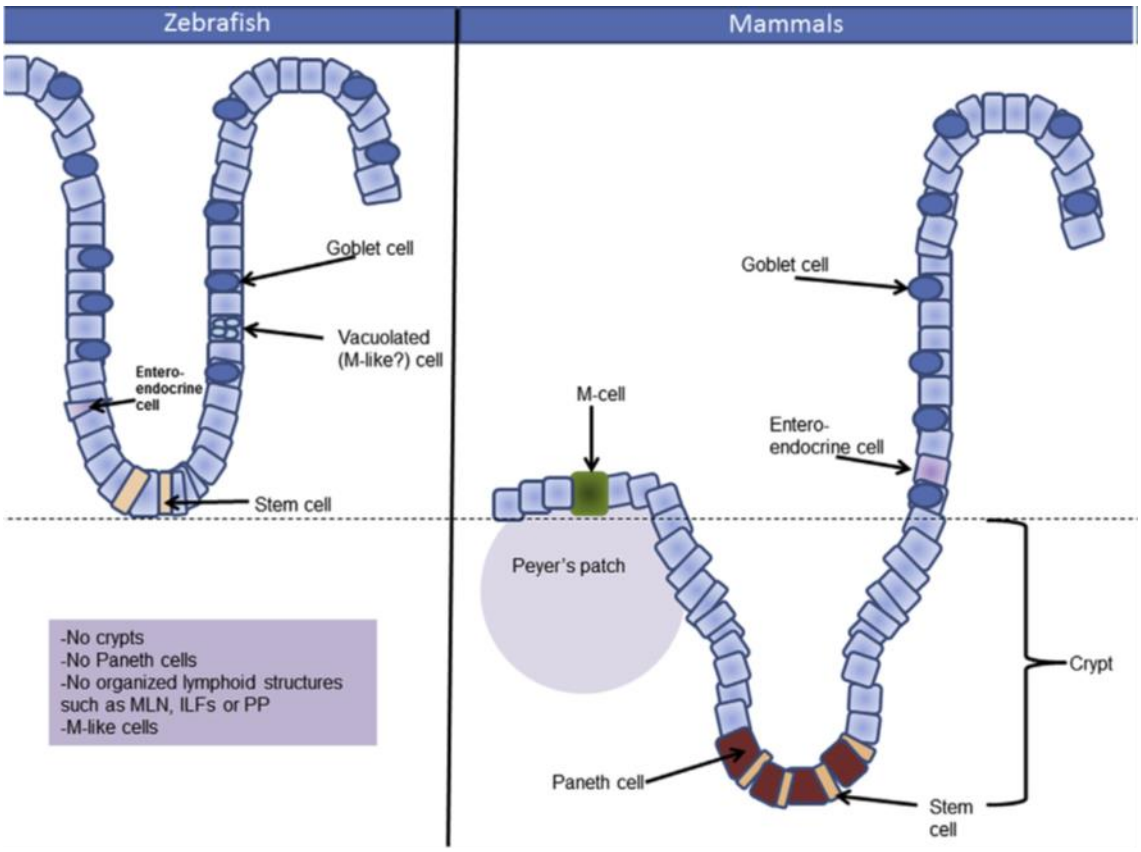


Figure 1-3: Difference in cell types and structures between the zebrafish and mammalian (small) intestines. Adopted from (Brugman, 2016).

The zebrafish gut microbiota is numerically dominated at all stages of the zebrafish life cycle by members of the bacterial phylum Proteobacteria, with the phyla Firmicutes and Fusobacteria also prevalent during larval and adult stages respectively. Few studies exist that addressed the effects of either MC-LR or *M. aeruginosa* on the gut microbiota in few organisms such as in rat, mice and the rhizosphere microbiota for terrestrial plants, however not in zebrafish. Besides that, it is important to understand and address, if the gut microbiota of domesticated lab-reared zebrafish is similar to zebrafish collected from their natural habitat or how the composition of the zebrafish gut microbiota community varies between zebrafish from different aquaculture facilities (Roeselers et al., 2011). According to Roeselers et al. (2011), the gut microbiota of laboratory-reared zebrafish is similar in composition to zebrafish collected recently from their natural habitat. They used *16S* rRNA gene sequence-based to compare gut bacterial communities in zebrafish collected newly from their natural habitat and with zebrafish that collected from different lab facilities in different geographic locations. Even the patterns of gut microbiota structure in domesticated zebrafish were varied across different lab facilities, but the gut microbiota membership in domesticated and recently collected zebrafish was similar with a common core gut microbiota. Another study by Lin et al. (2015) showed that the short-

term dietary exposure of MC-LR has made a significant decline in the composition of functional genes in rat gut. As they designed their study to reveal the shift of microbial functional genes in the gut of rat orally taken MC-LR. They used the Geo-Chip method and this way would detect a high diversity of bacterial and fungal genes involved in basic metabolic processes and stress resistance. Their results showed that the arrangement of functional genes was significantly changed in rat gut after one week of exposure to MC-LR. Also, they found some relatively enriched genes that are involved in carbon degradation including chitin, starch and limonene metabolism. These genes were mainly derived from fungal and bacterial pathogens. Furthermore, they found large amounts of significantly enriched genes relevant to degradation of the specific carbon compounds and aromatics. The enriched gene functions can be linked to acute gastroenteritis induced by MC-LR. Similarly, a study by Chen et al. (2015), as they studied the effects of sub-chronic MC-LR on gut microflora in different gut regions of mice. After intragastrical administration of MC-LR and using the Denaturing Gradient Gel Electrophoresis (DGGE) method to record and profile the shifting of the microbiota. Their results showed increases in the microbial species richness in caecum and colon. Additionally, MC-LR disturbed the balance of the gut microbiota and the MC-LR toxicological effect was varied among the gut regions of the mice. So, until now, little is known about the impact of *M. aeruginosa* or MC-LR on the gut microbial community, as it is important to know what will happen to the gut microbiota community in adult zebrafish after dietary exposure to the MC-LR and *M. aeruginosa*?

1.11. Zebrafish

Zebrafish are considered a perfect model for biomedical research including much lower husbandry costs than mammals. Zebrafish are easily housed in compact recirculating systems and have short generation times of approximately 3–5 months (Detrich et al., 1999). In addition, adult zebrafish have small size, which allow efficient and low-cost evaluation of all major organs on a limited number of slides (Fournie et al., 1996). Also, the small size of embryos minimizes the cost and waste volume for toxicant studies. Among vertebrates, the zebrafish's embryo offers optical clarity, allowing visual tracing of individual cell fates throughout organogenesis. A wide range of histochemical markers for gene expression and protein allows identification of essentially all cell types (Byrd and Brunjes, 1995; Connaughton et al., 1999; Cerda et al., 1998; Cox and Singer, 1999; Fritsche et al., 2000; Imboden et al., 1997; Kawai et al., 2001; Tsai et al., 2001; Van Nassauw et al., 1991; Van Raamsdonk et al., 1980; Wullimann and Rink et al., 2001;

Yelon et al., 1999). As a result of these advantages of zebrafish regarding molecular development studies and basic developmental biology, the zebrafish has emerged as the premier vertebrate model for clarification of the roles of specific genes and signalling pathways in development. (Spitsbergen and Kent, 2003). In addition, the number of studies that are adopting zebrafish is increasing and continues to show the power of the zebrafish model when investigating conserved pathways in gut epithelial homeostasis and inflammation. Furthermore, zebrafish can provide a perfect platform to develop molecularly targeted therapies by providing a high throughput screening tool in the search for novel compounds, which may reverse gene defects associated with intestinal diseases (Brugman, 2016).

Furthermore, zebrafish are an outstanding model for aquatic toxicology. Zebrafish produce large numbers of eggs, do not require high maintenance and have low background incidence of tumours. Besides that, zebrafish have rapid development and transparent chorion allow embryogenesis to be easily observed. All these characteristics of zebrafish make it an ideal organism for observing the effect of toxins on early development. (Black et al., 1985; Metcallfe and Sonstegard, 1985; Grizzle et al., 1988). Many previous studies have used zebrafish as a model to study the effects of MCs and /or *Microcystis* such as Oberemm et al. (1997); Oberemm et al. (1999); Best et al. (2002); Wang et al. (2005); Rogers et al. (2011) and Pavagadhi et al. (2012).

1.12. Hypotheses and Objectives

1.12.1. Hypotheses

A. Larval zebrafish hypotheses

- *M. aeruginosa* / pure MC-LR toxin may be a natural source of environmental estrogens
- Aqueous exposure of *M. aeruginosa* / pure MC-LR toxin (dose and time course relation) has an effect on gene expression profile for some target genes. Of particular interest are genes involved in vitellogenin 1 (*VTG1*); biotransformation genes [cytochrome P450 (*CYP1A1*), and glutathione-S-transferase 1 (*GST1*)]; oxidative stress group [catalase (*CAT*), superoxide dismutase 1 (*SOD1*) and glutathione peroxidase (*GPx*)] and liver protein phosphatase (*PPP1ca*).
- *M. aeruginosa* and pure MC-LR toxin cause histopathological changes in the liver's hepatocytes.
- *M. aeruginosa* and pure MC-LR toxin have histopathological effects on intestinal mucosa.
- *M. aeruginosa* and pure MC-LR toxin have different effects on larval zebrafish's gene expression, morphology and histopathology.

B. Adult zebrafish hypotheses

- Feeding exposure of *M. aeruginosa* / pure MC-LR toxin (dose and time course relation) affect gene expression profile of some target genes in liver. Of particular interest are genes involved in oxidative stress group [catalase (*CAT*), superoxide dismutase 1 (*SOD1*) and glutathione peroxidase (*GPx*)]; liver protein phosphatase (*PPP1ca*) and biotransformation genes [cytochrome P450 (*CYP1A1*) and glutathione-S-transferase 1 (*GST1*)].
- *M. aeruginosa* and pure MC-LR toxin cause histopathological changes in the liver's hepatocytes.
- *M. aeruginosa* and pure MC-LR toxin have histopathological effects on intestinal mucosa.
- *M. aeruginosa* and pure MC-LR toxin cause histopathological changes in the trunk kidney.
- *M. aeruginosa* and pure MC-LR toxin cause changes in the hepatocytes nucleus size.
- *M. aeruginosa* and pure MC-LR toxin will affect the gut microbial community.

1.12.2. Objectives

The overall aim was to investigate the toxicity of *M. aeruginosa* and MC-LR in zebrafish *Danio rerio* by addressing the following specific novel objectives:

- The first objective (chapter 2) was to investigate the effect of aqueous exposure of *M. aeruginosa* and MC-LR on dose and time manner on zebrafish larvae by evaluating gene expression profile for specific target genes and examining the histopathological changes for liver and gut.
- The second objective (chapter 3) was to investigate the effect of sub-lethal dietary exposure of *M. aeruginosa* and MC-LR on adult zebrafish by evaluating gene expression profile for specific target genes for liver and histopathological investigation for liver, kidney and gut.
- The third objective (chapter 3) was to investigate the effect of sub-lethal dietary exposure of *M. aeruginosa* and MC-LR on gut physiology in adult zebrafish. Changes in the gut microbial community were assessed along with tissue morphology (histopathology).

Chapter Two

Effects of *Microcystis aeruginosa* and the toxin microcystin-LR on target gene expression profiles and histopathological changes in larval zebrafish *Danio rerio*

2.1. Introduction

MC-LR and *Microcystis* could accumulate in aquatic organisms and represent a health hazard to animals and humans, particularly fish. MC-LR could affect growth, reproduction and embryonic development (Zhang et al., 2008; Chen et al., 2009; Campos and Vasconcelos, 2010). The first main target for MC-LR to attack and accumulate in fish is mainly the liver (Deblois et al., 2011; Papadimitriou et al., 2012). Previous studies reported that when fish were exposed to MC-LR or *Microcystis* during early life stages, the toxicity of purified MC-LR was less than *Microcystis*. Therefore, it is crucial to consider the effects of both *Microcystis* and MC-LR in larval fish (Oberemm et al., 1997; Oberemm et al., 1999; Best et al., 2001; Palikova et al., 2007). According to the Malbrouck and Kestemont review on fish (2006), MC-LR could cause morphological abnormalities such as small head or curved body and has the potential to disrupt embryonic hatching or growth rate. Additionally, MC-LR affects the heart rate and the physiological hepatocytes structure.

Zebrafish embryos and larvae have been reported to absorb and be affected by MCs, especially MC-LR through concentration-dependent growth reduction and the activation of the biotransformation system (Oberemm et al., 1997; Wiegand et al., 1999). However, numerous studies regarding MCs effects on early life stages of fish have documented the toxicity of MCs during the embryo stage (before hatching). These studies assessed the effects of MCs on fish larvae before hatching and examining the effects was done while larvae were grown in clean water (Malbrouck and Kestemont, 2006).

Vitellogenin gene (*VTG1*) expression in zebrafish larvae has been investigated and evaluated in the context of the toxicity of *M. aeruginosa* or MC-LR. To date, just one study by Rogers et al. (2011) had found induction of the *VTG1* gene when they exposed the zebrafish larvae to *M. aeruginosa* or MC-LR in three doses (0, 100 and 1000 µg/L) for 96 hpf. Their results showed that the *VTG1* gene was upregulated from 19.2-fold to >100-fold at doses of 100 and 1000 µg/L, with *Microcystis*, but no induction with MC-LR in the same treatments. These results suggest that *Microcystis* has an estrogenic effect, but not the same with MC-LR. Moreover, there are not any other studies or any other experiments that exist for comparison with Rogers et al. (2011) paper. However, the level of *VTG1* induction is consistent with the exposure to 17 β-estradiol (E2) and zebrafish larvae are considered a crucial tool to assist whether there is an estrogenic effect or not for some substances that will maybe cause *VTG1* induction or

reduction. The gap in information is what will happen to the *VTG1* gene expression after sub-lethal exposing zebrafish larvae to low concentrations of *Microcystis*/ MC-LR?

Few previous studies reported the effects of MC-LR and *Microcystis* on oxidative stress, biotransformation and protein phosphatase in early life stages in zebrafish. A study by Weigned et al. (1999) investigated the uptake of MC-LR in the different life stages of zebrafish through using ^{14}C-labelled MC-LR (doses 0.1, 0.5, 1, 2 and 5 μg MC-LR/L). Some of their results showed that GPx enzyme activity increased at 0.5 μg MC-LR/L. Currently, there are not any studies regarding zebrafish larvae with *M. aeruginosa* or MC-LR and biotransformation gene expression profile. A study by Tzima et al. (2017) showed that after exposing zebrafish larvae to 50 and 500 μg/L MC-LR for four days, the results revealed 40% reduction of PP2A enzyme activity in comparing to the controls, which may indicate the early effects of MC-LR.

The previous studies showed that fish larvae could be especially vulnerable to MC-LR exposure. A better understanding of mechanisms of MC-LR toxicity during this stage is needed. Also, understanding and filling the gaps regarding gene expression profiles for the biotransformation related genes (*CYP1A1, GST1*), oxidative stress genes (*SOD1, CAT, GPX*), protein phosphatase pathway (*PPP1ca*), vitellogenin gene (*VTG1*) and histopathological investigation for zebrafish larvae are required. Therefore, the objectives of the work in this chapter are to investigate the aqueous exposure effects of *M. aeruginosa* or MC-LR on the expression of specific genes related to the detoxification pathway, oxidative stress, vitellogenin and protein phosphatase. This research project also aimed to investigate the histopathological effects of MC-LR or *M. aeruginosa* on larval zebrafish as a function of dose and time response.

2.2. Materials and Methods

2.2.1. Zebrafish larvae

All the experimental 72 hours post fertilization hatched zebrafish larvae were obtained from the zebrafish facility at Heriot-Watt University. The zebrafish stock was originally obtained from Edinburgh University, Edinburgh, UK. The zebrafish facility was maintained under routinely approved animal welfare protocols. Adult zebrafish were fed three times daily with fish dry food particles in the morning, then two times with live brine shrimp nauplii (*Artemia* spp.) and the photoperiod was 12:12 hours light and dark cycle. The temperature should be between (27-29 ˚C). The maintenance diet should be 0.5-2% body weight per day. The broodstock was fed more before the spawning. Zebrafish are gastric, so the most efficient feeding schedule was a small amount of food regularly. The water quality parameters were measured daily for the pH and temperature and weekly for chlorine, ammonia, nitrite and nitrate levels. The acceptable levels are (7.0 - 7.5) for pH, (0.0 - 2.0 mg/L) for chlorine, (0.0 – 0.5 mg/L) for ammonia (0.0 – 0.3 mg/L) for nitrite and (0.0 – 4.5 mg/L) for nitrate. These parameters levels were measured by using specific measurements stripes, which were bought from King British, Lincolnshire /UK and the chlorine strips were bought from Cam lab, Cambridge /UK. The spawning was carried out routinely two times per week and all the embryos were cleaned and kept at room temperature in 50 mm Petri dishes.

2.2.2. Microcystin-LR stock

MC-LR (5 mg) lyophilized material was purchased from (Biorbyt, Cambridge /UK) with a purity around 98-99% and dissolved in 1 ml milliQ water to obtain a concentration of 5 mg/ml. The 1-2% impurities are ammonium acetate and some salts. A stock solution concentration of 5 mg MC-LR / ml Milli-Q water was prepared.
To check the concentration of MC-LR that had been ordered, the ELISA kit was used. From the MC-LR stock, sub-stock solutions were prepared, which had 2 ppb concentration to mimic one of the ELISA kit standards, i.e., 0, 0.1, 1, 2 and 5 ppb. The ELISA kit directions were followed to analyse the samples.
The results from the ELISA confirmed that the concentration of MC-LR stock solution was what had been expected, which was 5mg /1ml Milli-Q water (Figure 2-1).

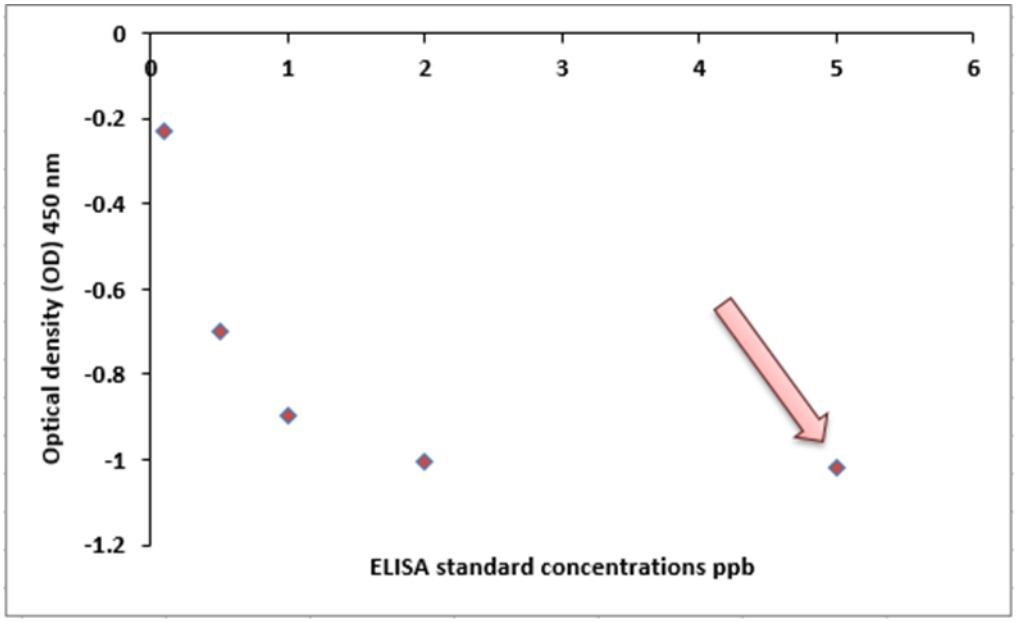


Figure 2-1: The reading data between the standard concentrations of the ELISA kit and the optical density (OD) 450 nm. The arrow pointed to where the similarity between the higher standard concentration of the ELISA kit and the concentration of MC-LR stock solution, which was consistent with the supplier's report 5mg.

2.2.3. Culturing and counting cyanobacteria *M. aeruginosa*

Cyanobacteria *M. aeruginosa* (1450/10 toxic strain) was required for use in ecotoxicology exposure assays. *M. aeruginosa* was purchased from (CCAP, Argyll / UK) and was cultured and harvested at Plymouth University, UK. For culturing *M. aeruginosa*, BG11 media was used from (Biorbyt, Cambridge /UK), incubated at 22 ˚C, 12:12 light/dark hours photoperiod in incubator model Snijder Economic Lux Climate chamber that had light intensity 10.5 µE $m^{-2}S^{-1}$ and the lights were all around the cultures. Cell counting was performed by using the hemocytometer (Figures 2-2 & 2-3) to count the total number of *M. aeruginosa* cells in the WBC squares which are the four big squares that are subdivided into 16 small squares. When cultures had reached exponential phase, which is around 5-7 days from the first day of starting the culture and thus when they could be harvested for lyophilisation. Then, the average of three readings from each culture each day was taken to record the data and to calculate the average concentration of cells (also known as cell density, i.e., cells / ml). Finally, drawing a graph (Figure 2-4) as the X-axis is days and the Y-axis the no. of cells per ml (mean ± standard deviation). The initial storage of the harvested liquid samples was at - 20 ˚C.

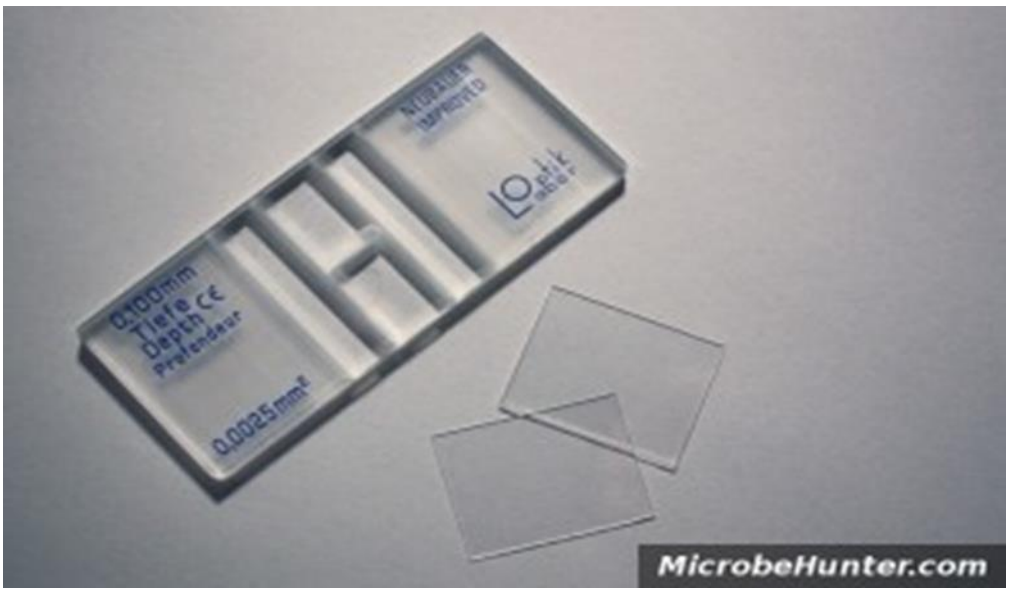


Figure 2-2: Hemocytometer (The counting chamber) adopted from http://www.microbehunter.com/2010/06/27/the-hemocytometer-counting-chamber/

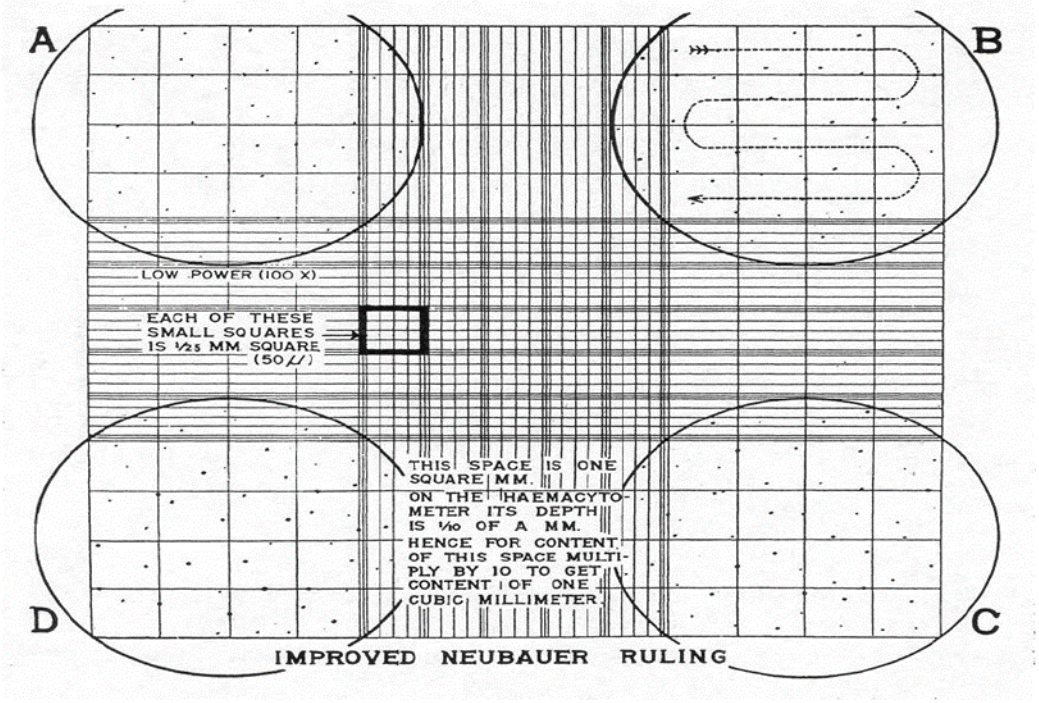


Figure 2-3: Hemocytometer, the A, B, C, D: WBC squares adopted from http://www.free-ed.net/sweethaven/MedTech/Hematology/lessonMain.asp?iNum=0502

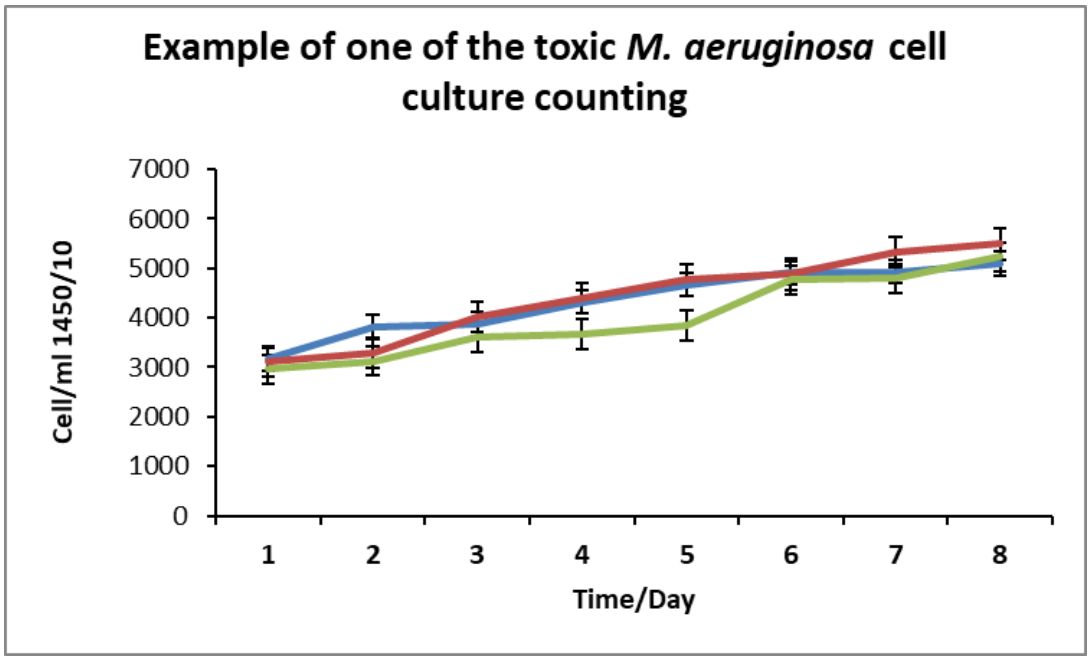


Figure 2-4: Example of one of the toxic *M. aeruginosa* cell culture counting. The results represented by (mean ± S.E. / three readings for each replicate).

2.2.4. Storing material for lyophilisation (freeze-drying) of *M. aeruginosa*

When the cultures had reached exponential growth phase, which is the point when most microcystin would be produced estimated to be around 5-7 days growth. Then, the cultures were evaluated based on cell count data and centrifuge tubes (size 50 ml) were filled with the culture and stored at -20 ˚C. Then, lyophilisation process was performed by freezer dryer machine (Figure 2-5 A) to prevent degradation of endogenous (cells) and exogenous (liquid medium) toxins. The frozen liquid samples of *M. aeruginosa* were placed in patches inside the lyophilizing machine after removing the caps and covered with parafilm that had some handmade pores in them. Then, the frozen samples were left inside the machine for nearly five days. After that, all the dried samples were mixed, weighed and stored at -20 ˚C. Finally, a microcystin ELISA kit form (Biorbyt, Cambridge /UK) was used to quantify MC-LR in the lyophilized samples of *M. aeruginosa*. A stock solution concentration 5.675mg *M. aeruginosa* lyophilized cells / 0.5 ml DI water was prepared and then serial dilutions (1:10, 1:100 and 1:1000) were prepared from the main stock. By following the kit's direction, the ELISA results showed that the MC-LR concentration in the 1 mg of the lyophilized *M. aeruginosa* main stock solution was 4 ppb (Figure 2-5 B). Depending on the ELISA results, a stock solution concentration of 0.025 µg *M. aeruginosa* lyophilized cells / 1µl Milli-Q water was prepared, which was required for the different experiments to prepare the different doses.

Figure 2-5 A: The freezer dryer machine at Heriot-Watt University.

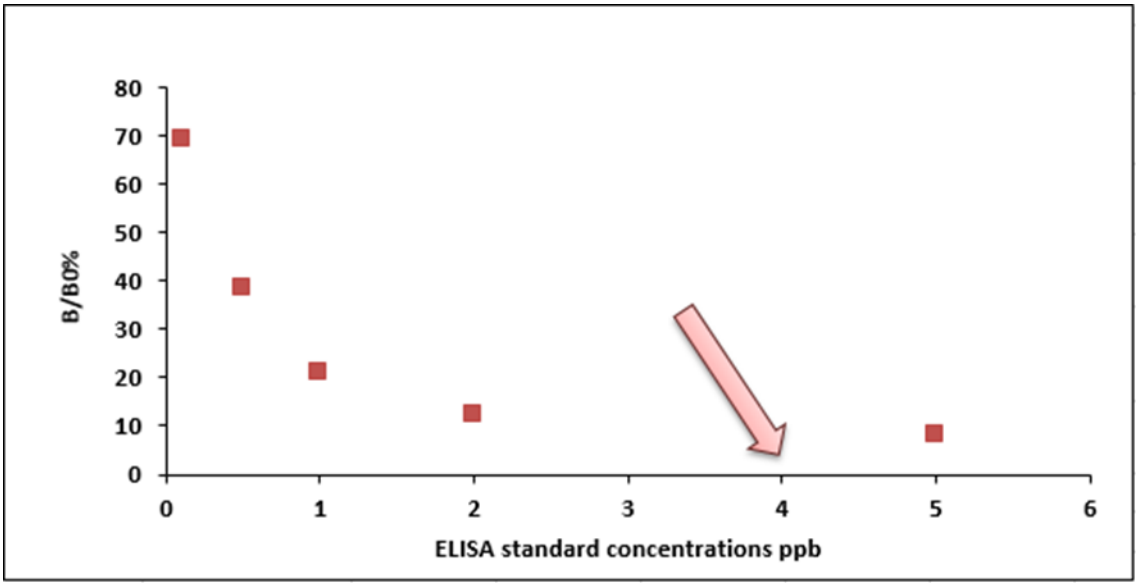


Figure 2-5 B: The reading data between the standard concentrations of the ELISA kit and the value of B/B0%. B/B0% value is dividing the optical density (OD) of the testing sample on the optical density of the control and multiply by 100). The arrow pointed to the concentration of MC-LR in 1 mg of the lyophilized *M. aeruginosa* stock solution, i.e., 4 ppb.

2.2.5. Experimental design

2.2.5.1. Experiment 1: MC-LR / *M. aeruginosa* concentration relationship

Zebrafish larvae (age 72 hours post fertilization) were sub-lethally exposed for 96 hours to MC-LR or lyophilised *M. aeruginosa* at concentrations (5, 25, 50, 100, 200 and 400 μg MC-LR/L). Three replicates were performed for each dose and 25 larvae were used for each concentration replicate. These doses were chosen to mimic the real environmental concentrations, as there were three low doses (5, 25 and 50 μg MC-LR/L) and three higher doses (100, 200 and 400 μg MC-LR/L). The endpoints of this experiment were mortality, deformity, gene expression and histopathology for liver and gut. The mortality and deformity were measured by using the dissecting microscope model Meiji Techno RZ (Figure 2-6) to check the larvae's heartbeat, morphology and movement during and at the end of the experiment.

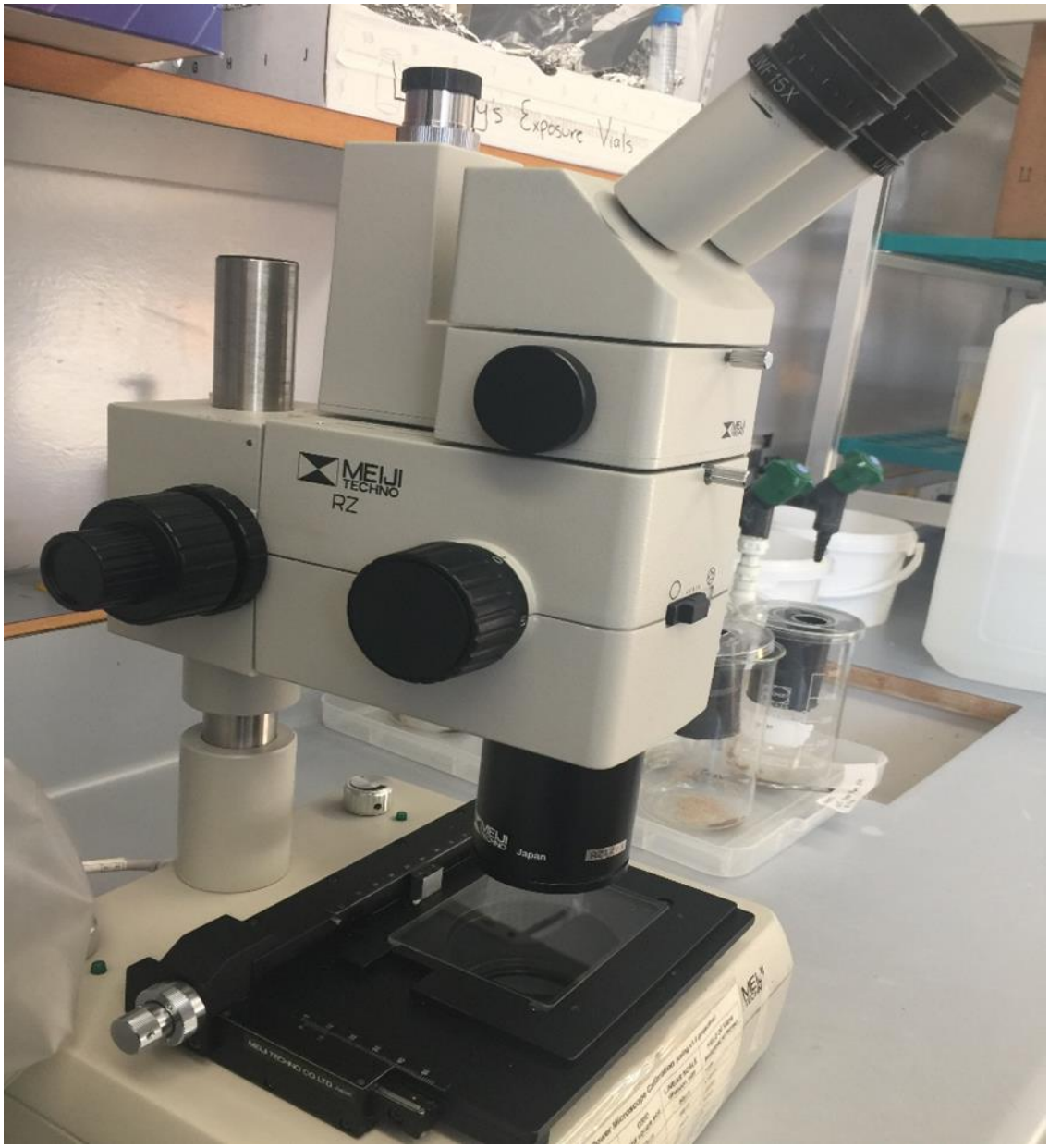


Figure 2-6: The dissecting microscope model Meiji Techno RZ.

2.2.5.2. Experiment 2: MC-LR / *M. aeruginosa* time relationship

Zebrafish larvae (age 72 hours post fertilization) were sub-lethally exposed for up to 96 hours to MC-LR or lyophilized *M. aeruginosa* doses (5 and 50 µg MC-LR/L). As, a low dose 5 µg MC-LR/L and a slightly higher dose 50 µg MC-LR/L were chosen to mimic the real environmental conditions and to avoid the mortality in order to investigate the changes in gene expression during the different sampling time (4, 24, 48, 72 and 96 hours). Three replicates were performed for each concertation and for each time point. Also, 25 larvae were used for each concentration & each time replicate. The endpoint of this experiment was the evaluating changes in gene expression for target genes in relation to the exposure time.

2.2.6. Target genes, housekeeping genes and the efficiency

The target genes for whole larvae samples were vitellogenin 1 (*VTG1*), catalase (*CAT*), superoxide dismutase1 (*SOD1*), glutathione peroxidase (*GPx*), glutathione-S-transferase 1 (*GST1*), cytochrome P450 (*CYP1A1*) and protein phosphatase 1ca (*PPP1ca*). The two reference genes were *β-actin* and glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) and the selected reference gene for analysing the Q-PCR data was *GADPH*. The Q-PCR efficiencies for the reference genes and the target genes were between 91% and 110% (Table 2-1).

Table 2-1: Zebrafish (*Danio rerio*) specific primers for target genes (*VTG1*, *SOD1*, *CAT*, *GPX*, *CYP1A1*, *GST1* and *PPP1ca*) and housekeeping genes (*β-actin* & *GADPH*).

Gene	*Reference sequence Number	Forward (5′–3′)	Reverse (5′–3′)	Product length (bp)	Annealing temperature (°C)
VTG1	NM_001044897.2	ATCAGTGATGCACCTGCCCAGATTG	ACGCAAGAGCTGGACAAGCTGAA	117	60
SOD1	NM_131294.1	ACCGGCACCGTCTATTTCAA	AGCATGGACGTGGAAACCAT	105	55
CAT	NM_130912.1	CAAGGTCTGGTCCCATAAA	TGACTGGTAGTTGGAGGTAA	227	58
GPX	NM_001007281.2	AGGCACAACAGTCAGGGATT	CAGGAACGCAAACAGAGGG	241	58
CYP1A1	NM_131879.1	AGGACAACATCAGAGACATCACCG	GATAGACAACCGCCCAGGACAGAG	174	60
GST1	NM_001045060.2	TCGTCTACCAGCGCATGTTT	CTCCAGGTATCCCTCCCACA	164	60
PPP1ca	NM_214811.2	AAGAGAAAAGGGGCTTAGAGGAT	CAGGAAACGGTTAATGTGGTACA	137	60
β-actin	NM_131031.1	ACACAGCCATGGATGAGGAAATCG	TCACTCCCTGATGTCTGGGTCGT	138	60
GADPH	NM_0011151114	CTGGTGACCCGTGCTGCTT	TTTGCCGCCTTCTGCCTTA	150	60

*Reference sequence numbers from NCBI, and product length in base pairs (bp).

2.2.7. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA for whole zebrafish larvae that were stored at -80 ˚C was extracted by using an RNeasy Mini Kit for animal tissue (Qiagen, Hamburg / Germany) from samples of 25 larvae and manually homogenised with the lysis buffer. The next steps included more tissue break-up by using a QiaShredder column (Qiagen, Hamburg / Germany), then 15 min DNase treatment. RNA was eluted into 30 µl nuclease-free water and the quality and the concentration for the total RNA was determined by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer) (Figure 2-7). As, the Optimum quality and concentration values for spectrophotometry of RNA: > 100 ng/µl, ratio 260/280 - 1.9-2.2 and ratio 260/230 – 1.5-2.2 (Figure 2-8). Then, the samples were diluted to 100 ng/µl of total RNA and 800 ng for each sample were used to synthesise complementary DNA (cDNA) following the manufacturer's protocol (ImProm-II™ Reverse Transcription System; Promega), with deoxynucleotide mix and hexanucleotide primers (Sigma-Aldrich). cDNA was synthesised using the following conditions: annealing at 25 ˚C, extending at 42 ˚C and heat-inactivating transcriptase at 70 ˚C (Gene Amp PCR System, 9700; Applied Biosystems) (Figure 2-9). Finally, cDNA was stored at −20 ˚C until quantitative reverse transcriptase–PCR (qRT–PCR) gene expression analysis.

Figure 2-7: The NanoDrop 2000, ND-1000 Spectrophotometer (Thermo Scientific).

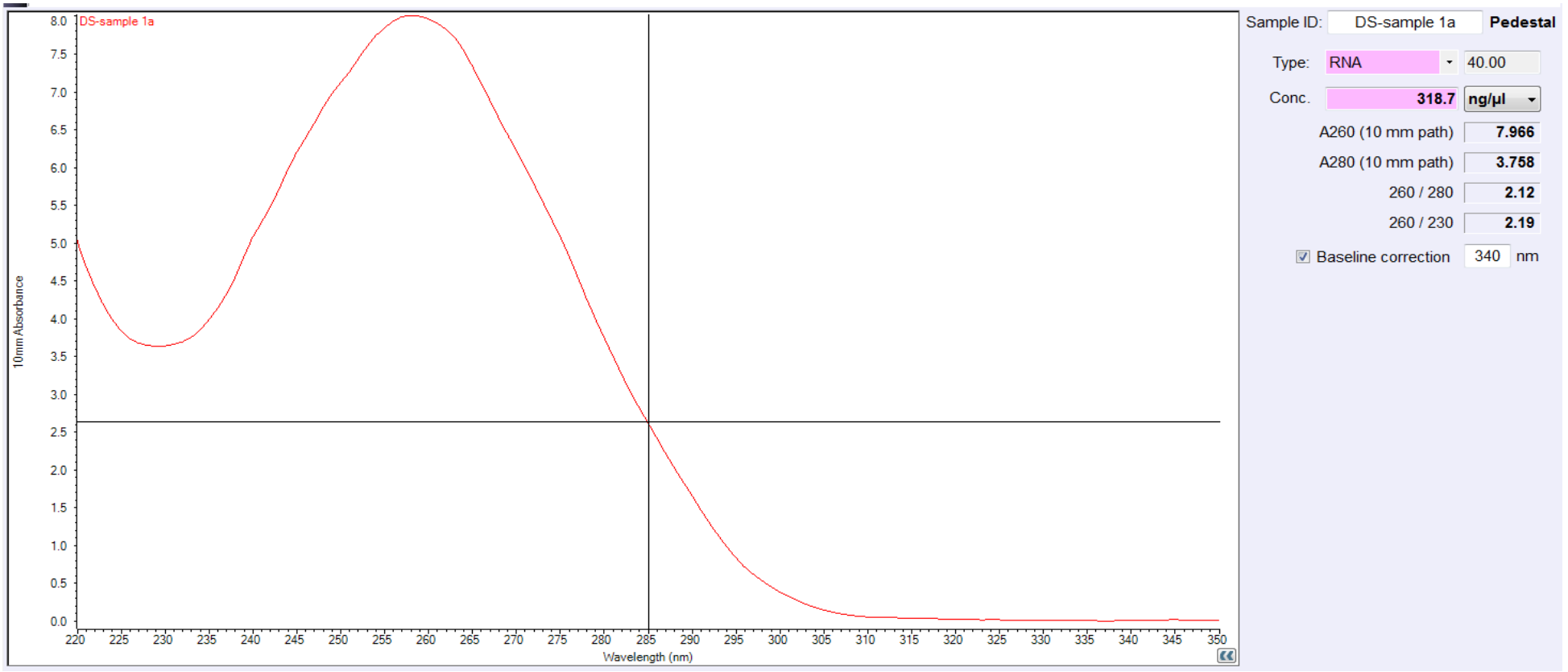


Figure 2-7: Example outputs from the nanodrop machine for one of the present study samples. As, the Optimum quality and concentration values for spectrophotometry of RNA: > 100 ng/µl, ratio 260/280 - 1.9-2.2 and ratio 260/230 – 1.5-2.2. The outcomes in the right side showing perfect values for the RNA concentrations and the ratios 260/280 and 230/260.

Figure 2-9: Gene Amp PCR System, 9700; Applied Biosystems.

2.2.8. Quantitative reverse transcriptase–PCR (qPCR)

Nearly all of the primers in (Table 2-1) were designed, selected and verified by using ZFIN (the zebrafish information network) by myself, Ph.D. researcher Dalia Sabrei, except *VTG1*, *GADPH* and *β-actin,* which were taken from previous studies (Rogers et al., 2010; Li et al., 2011). Then, the required primers for the specific target genes were purchased from Eurofins MWG (Operon, Ebensburg, Germany) and by following the manufacturer's protocol, the lyophilised primers were reconstituted with RNase-free water to give a final concentration of 100 μmol. Following this, the primers were mixed with SYBR Green JumpStart TaqReadyMix to give a final reaction concentration of 375 nmol in 20 μL of total volume. By using the StepOne Real-Time PCR System machine (Applied Biosystems) (Figure 2-10 A), the fluorescence was detected over 40 cycles, cycling conditions of 94 ˚C for denaturing, primer-specific annealing at 55–60 ˚C (Table 2-1) and extension at 72 ˚C (Reinardy et al., 2013). Some example outputs of gene expression of *SOD1* and *CYP1A1* for the present study showed in (Figures 2-10 B & C).

Figure 2-10 A: The StepOne Real-Time PCR System machine (Applied Biosystems). (B) QPCR screen showed an example for one of running one of the choosing genes (*β-actin*).

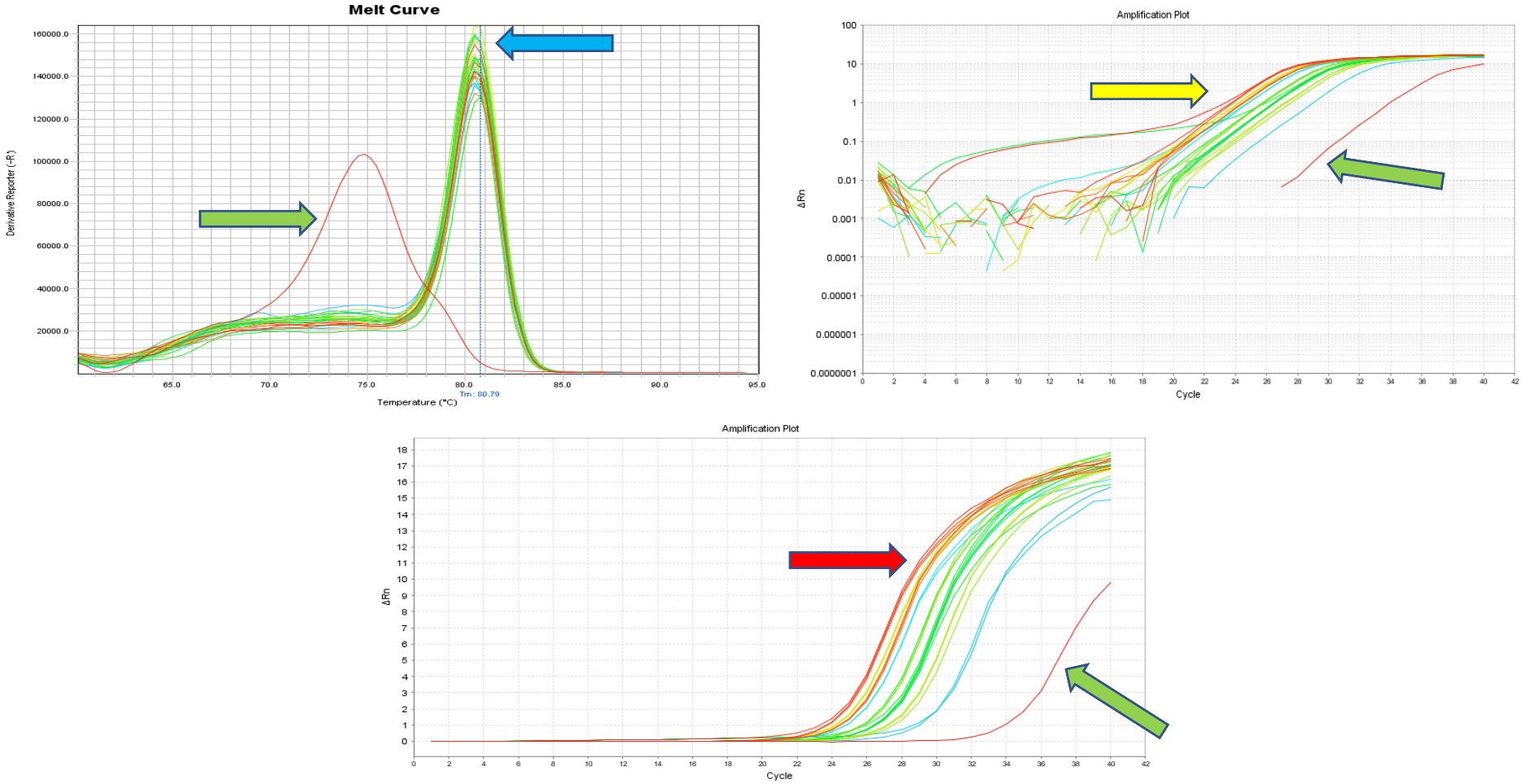


Figure 2-10 B: Example outputs from the QPCR machine for *SOD1* gene expression showing the melt curve (blue arrow), the log amplification plot (yellow arrow), the linear amplification plot (red arrow) and the green arrow showing the non-templet control (NTC), which is the negative control.

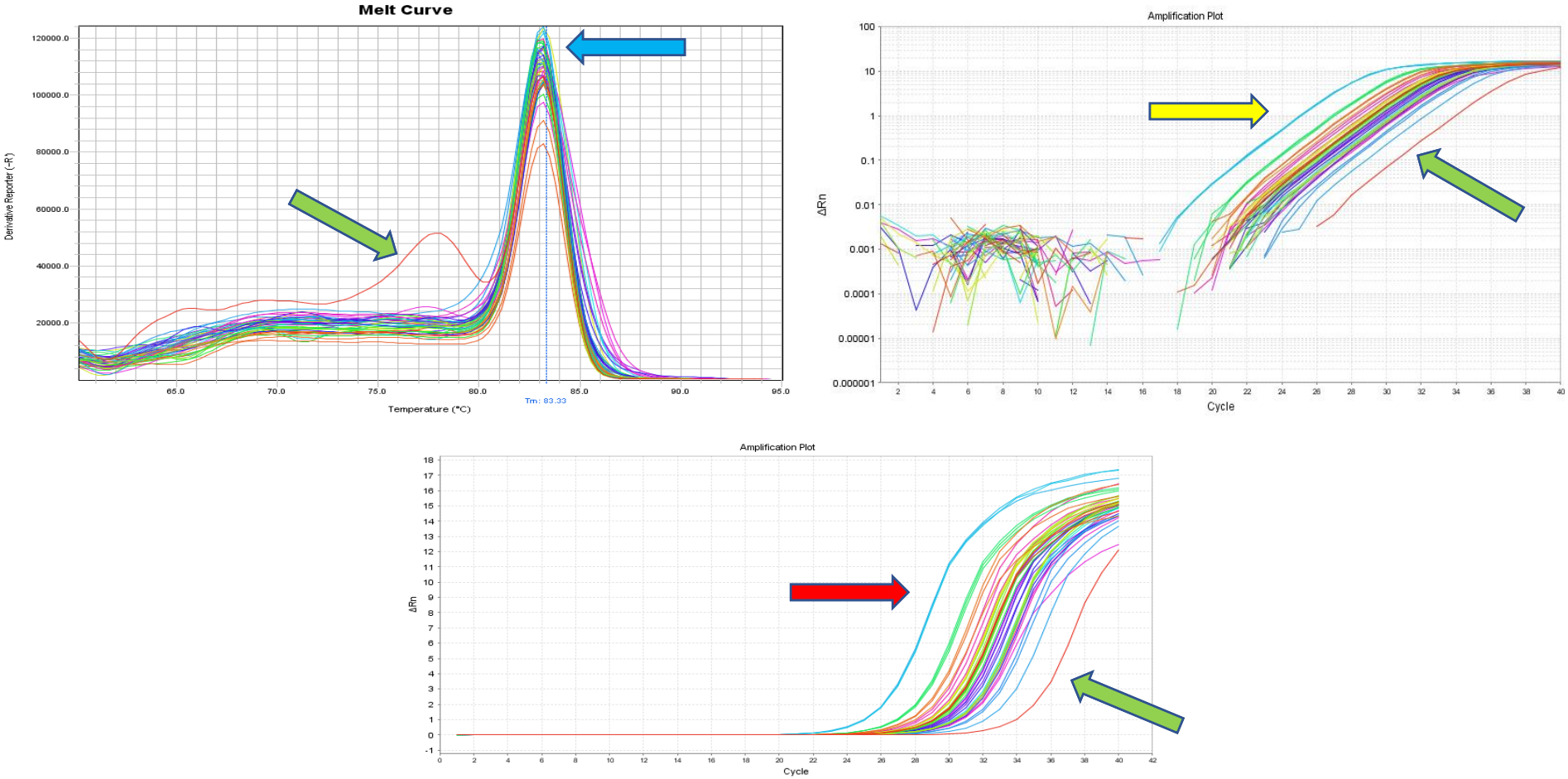
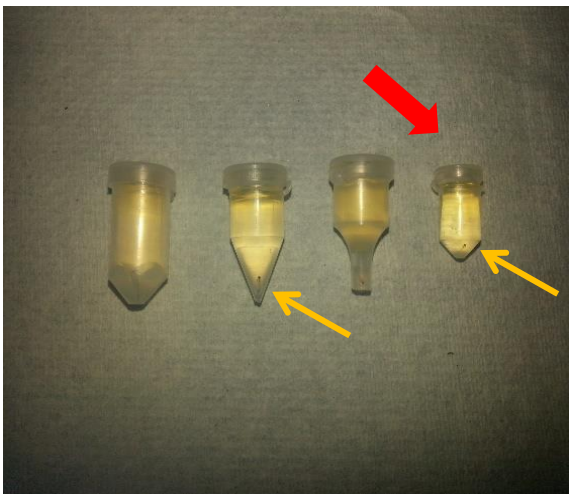


Figure 2-10 C: Example outputs from the QPCR machine for *CYP1A1* gene expression showing the melt curve (blue arrow), the log amplification plot (yellow arrow), the linear amplification plot (red arrow) and the green arrow showing the non-templet control (NTC), which is the negative control.

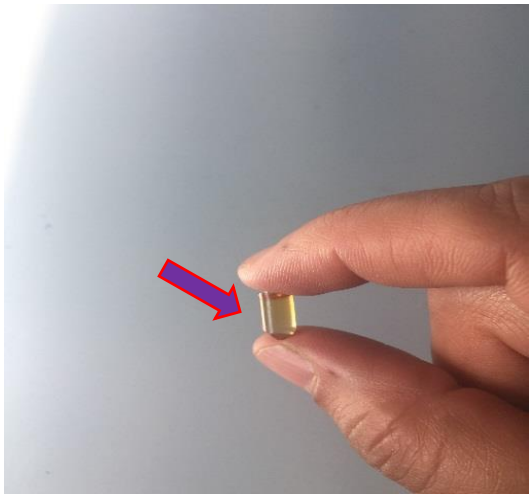
2.2.9. Histopathology

JB-4™ Resin kit (Sigma-Aldrich) for plastic histology procedure was used. The JB-4 embedding kit offers a unique polymer embedding material that gives a higher level of morphological detail than paraffin processed tissues. The whole larvae from the different treatments were kept in 10% Neutral Buffered Formalin (NBF) from Sigma Aldrich (Darmstadt, Germany) at room temperature overnight. The dehydration stage was completed at room temperate for the larval samples through serial dilutions of ethanol (70%, 90% and 100%) and after taking out all the NBF from the larvae samples. Next, the infiltration stage was performed at room temperature while avoiding exposure of the samples to either heat or direct light during the infiltration process. Following the manufacturer's manual, the infiltration was performed by preparing combined ascending mixing fluids by mixing equal amounts of absolute ethanol and the infiltration materials, i.e. JB-4 Solution A Monomer Benzoyl Peroxide and Plasticized Catalyst (Sigma-Aldrich). The embedding stage was performed at room temperature by mixing specific amounts of infiltration solution and JB-4 Solution B (Accelerator) (Sigma-Aldrich). The prepared capsules kind BEEM Capsules size 3 from (Agar Scientific) (Figure 2-11A) were left to dry at room temperature overnight in a stand way (Figure 2-11B). Then, sectioning was carried out for the plastic block (Figure 2-11C) with a glass knife on a microtome to get thickness (2 µm) (Figures 2-12 & 2-13). Finally, the slides were left to dry on the hot plate (temp~25 ˚C) and the toluidine blue protocol was followed to stain the sections. As other staining protocols were followed such as H&M (Haematoxylin & Eosin) and Methylene Blue, but the chosen protocol was Toluidine Blue. Moreover, the H&M protocol has long time processing and this affected the sections, as there were very small and delicate. However, Methylene Blue is very easy and fast, but the staining quality for the sections was not good. So, Toluidine Blue protocol was followed, as the slides were placed on a hot plate (temp~25 ˚C) and covered with toluidine blue stain for one minute, then the slides were rinsed carefully in slope way with tap water. After that, the slides were dipped quickly two times in 96% ethanol. Finally, the slides were placed in 100% ethanol for 30 seconds or up to 1 minute and were left to dry overnight. The endpoints that used were to measure the nucleuses sizes for the liver's cells, the liver and the gut morphology and histopathology changes. ImageJ software was used to measure the liver cell nucleus size.



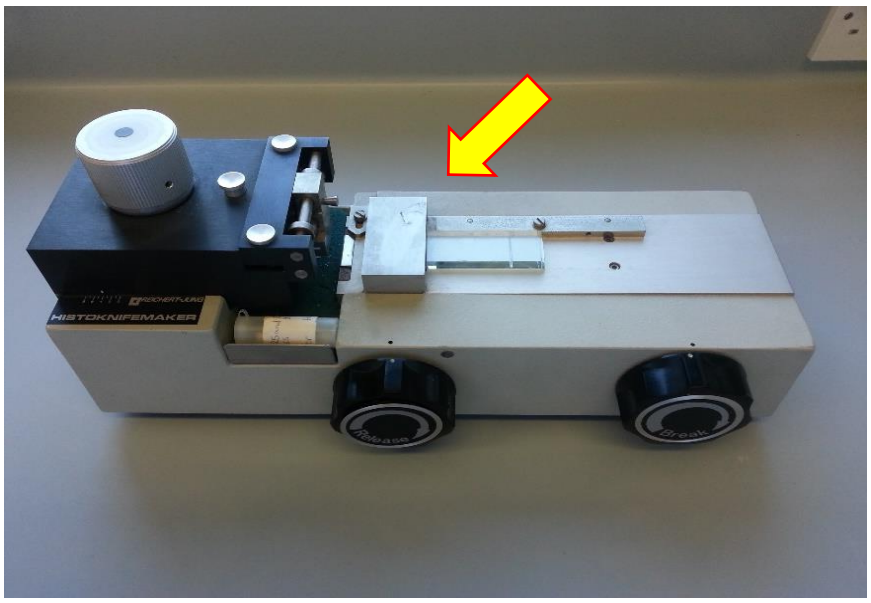
A

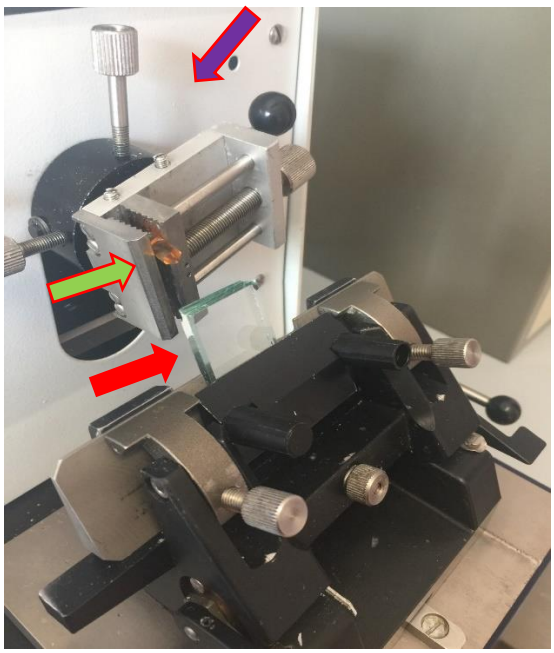
B



C

Figure 2-11: A and B) Showing different kinds and sizes of BEEM capsules. The red arrow showing the chosen BEEM capsule size 3 to embed the larval samples and the yellow thin arrow showing the position of the larvae. **C)** The purple arrow showing the plastic block after the embedding process.





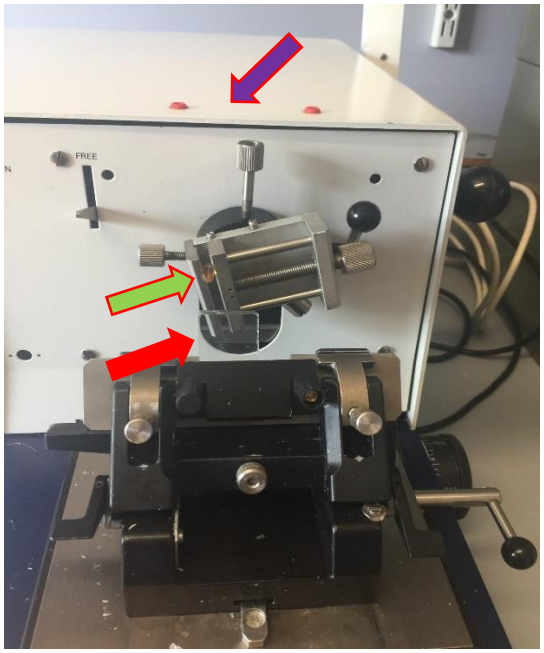


Figure 2-12: The yellow arrow pointing to the glass knife maker, the purple arrow showing a side of the microtome, the green arrow is the plastic block and the red arrow is the glass knife.

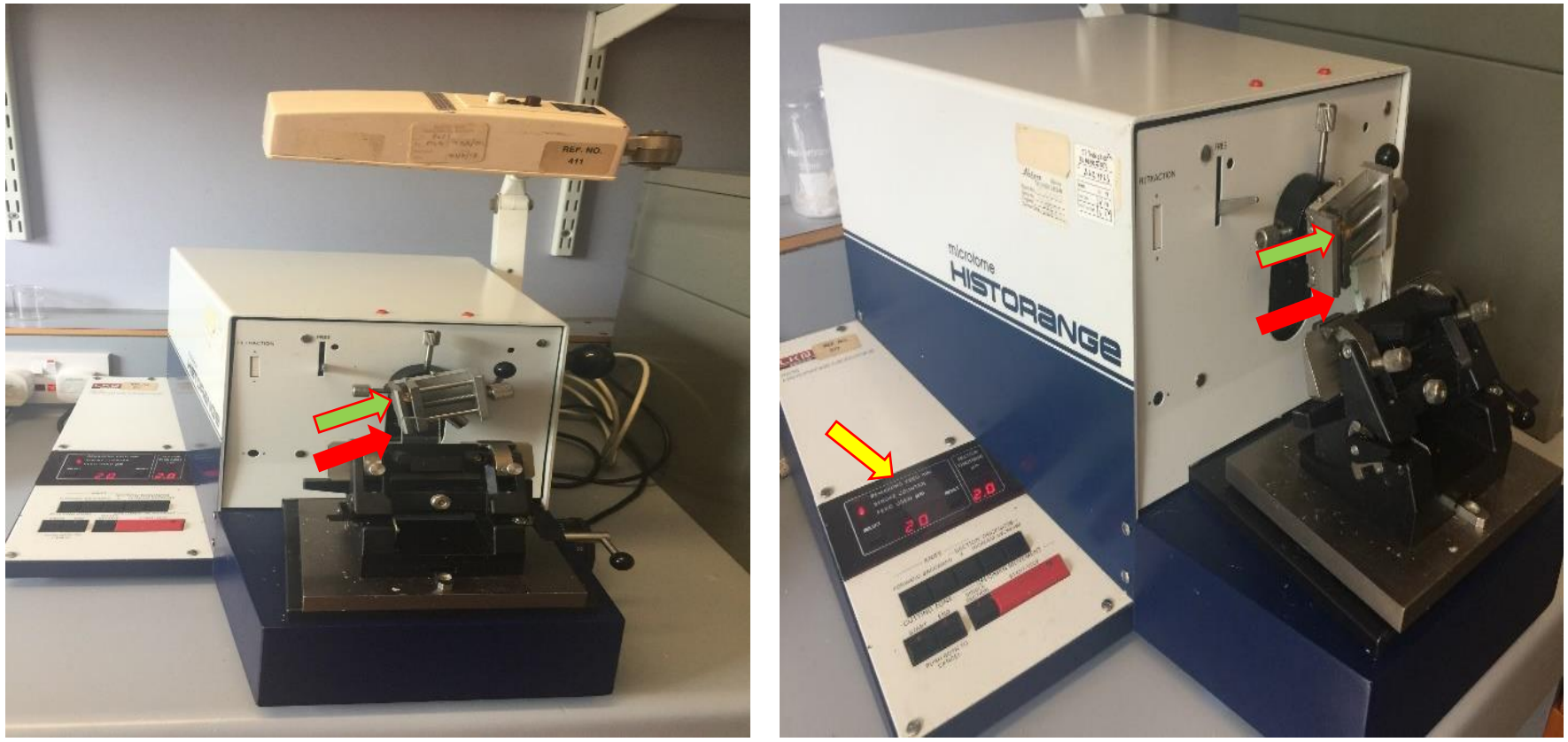


Figure 2-13: Showing the microtome from different angles. The yellow arrow shows the section thickness and the speed in the screen**.** The green arrow is the plastic block and the red arrow is the glass knife.

2.2.10. Statistical analysis

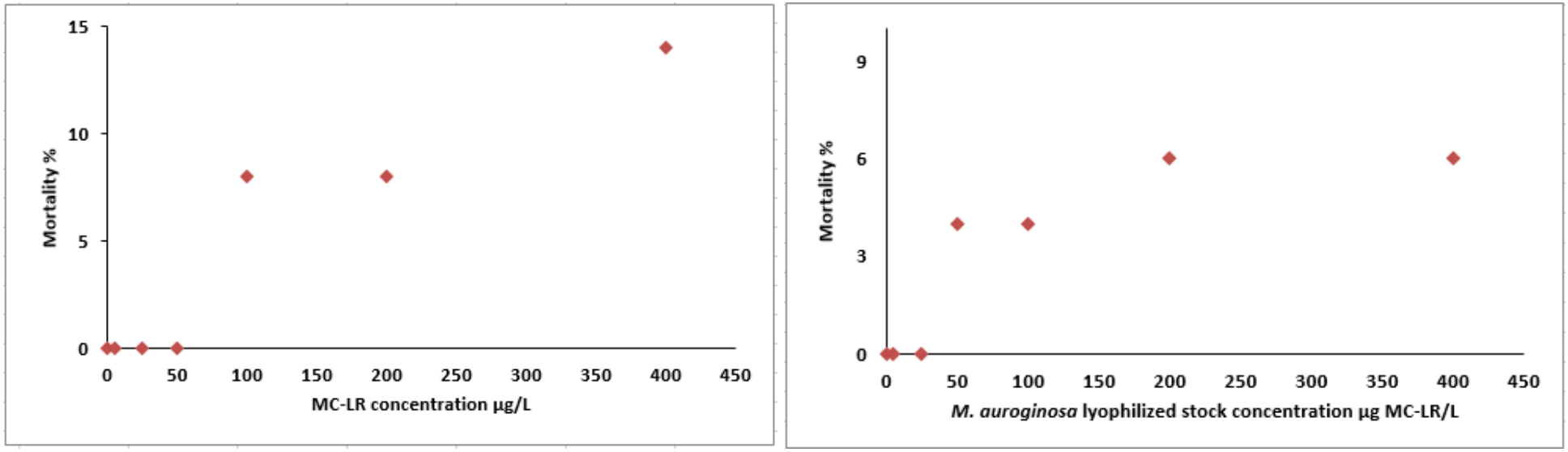
All the data, except the histology results, were statistically analysed using R-software (R Core Team, 2017), using Bartlett homogeneity of variance test was used to test the normal distribution of data. Then, one-way analysis of variance (ANOVA) was used to test for differences among different treatments in gene expression. Significant differences at ($p \leq 0.05$) and ($p \leq 0.001$) were compared using Tukey's multiple mean comparison tests. Data are presented as (mean ± standard error).

2.4. Results

2.4.1. Experiment 1: MC-LR / *M. aeruginosa* concentration relationship

2.4.1.1. Mortality and deformity

Mortality & deformity did not reveal significant effects with MC-LR / *M. aeruginosa* concentration relationship. The higher doses of MC-LR showed less than 20% mortality, and the higher doses of *M. aeruginosa* showed less than 10%. (Figures 2-14 A & B).



A **B**

Figure 2-14: (A) The mortality of zebrafish larvae after aqueous exposure to different concentrations of MC-LR and (B) *M. aeruginosa* for 96 hours, 3 replicates for each treatment / 25 larva for each replicate.

2.4.1.2. Gene expression

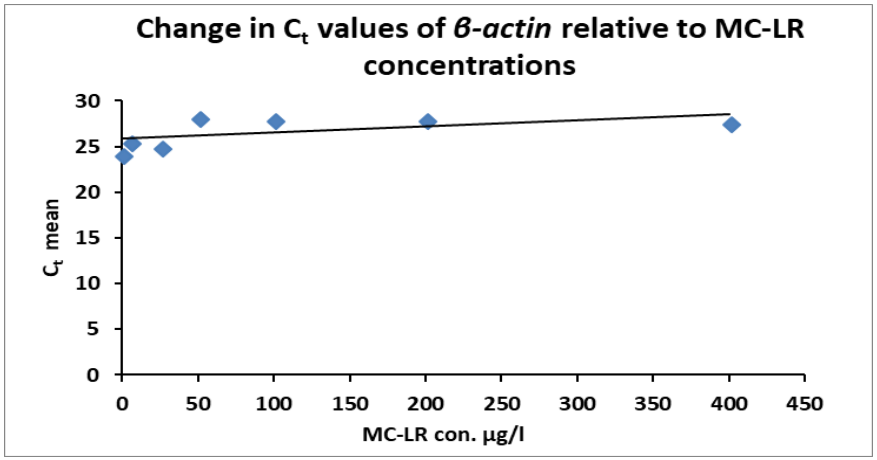
The gene expression profile revealed overall significant effects with MC-LR/ *M. aeruginosa* concentration relationship. The housekeeping gene is a relevant way to start. The effects of MC-LR on the reference gene *β-actin* showed increase in the C_t values with MC-LR concentrations. In contrast, the effects of lyophilised *M. aeruginosa* on the reference gene *β-actin* showed slight increases in the C_t values (Figures 2-15 A1&2), whereas, the effects of MC-LR and *M. aeruginosa* on the reference gene *GADPH* showed that the C_t values were nearly very close in comparison to C_t values of *β-actin* (Figures 2-15 B1&2). *GADPH* was chosen to be the housekeeping gene to analyse the Q-PCR data. These results indicated that *β-actin* expression was decreasing with MC-LR concentrations.

In the context of the target genes, *VTG1* showed significant responses with MC-LR / *M. aeruginosa* concentration relationship. The fish that were exposed to MC-LR showed that there was significant induction ($p \leq 0.05$) and ($p \leq 0.001$) to ~3 fold of *VTG1* with the higher doses (Figure 2-16 A). On the other hand, the group of fish that were exposed to the lyophilized *M. aeruginosa* at the same doses had significantly down-regulated *VTG1* (Figure 2-16 B).

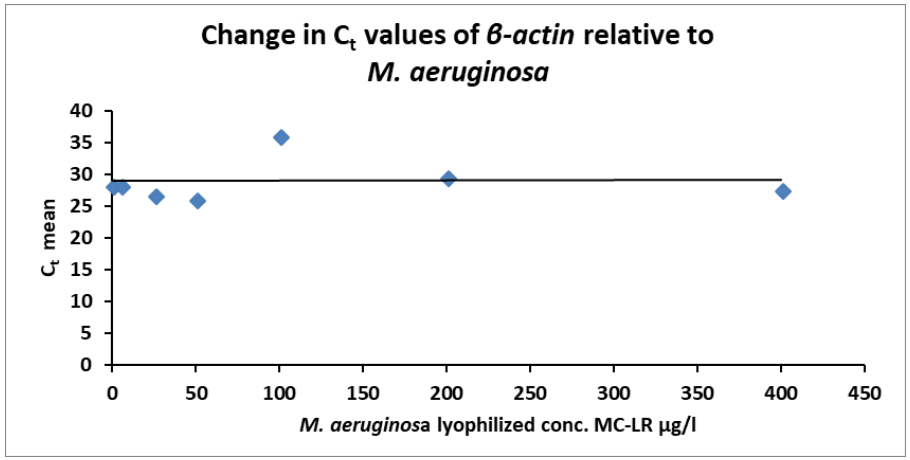
Oxidative stress-related genes showed significant responses with MC-LR / *M. aeruginosa* concentration relationship. Oxidative stress-related genes involved in resolving oxidative stress relations with exposure to MC-LR and *M. aeruginosa* (*CAT, SOD1* and *GPx*). Overall, there was slightly up-regulated with MC-LR lower doses, then started to be significantly ($p \leq 0.05$) and ($p \leq 0.001$) down regulations in the higher concentrations (Figures 2-17 to 2-19 A). In contrast, there were significantly down regulations with increasing the concentrations of *M. aeruginosa* (Figures 2-17 to 2-19 B).

Biotransformation related genes showed significant responses with MC-LR / *M. aeruginosa* concentration relationship. The results of MC-LR showed that there was the significant induction of *CYP1A1* and *GST1* with some MC-LR concentrations then significant down-regulated in the higher doses (Figures 2-20 & 2-21 A). Furthermore, *CYP1A1* induced significantly with the lower concentrations of *M. aeruginosa* and gradually down-regulated in the higher concentrations (Figure 2-20 B). However, *GST1* showed overall significant down-regulation ($p \leq 0.05$) and ($p \leq 0.001$) with increasing concentrations of *M. aeruginosa* (Figure 2-21 B).

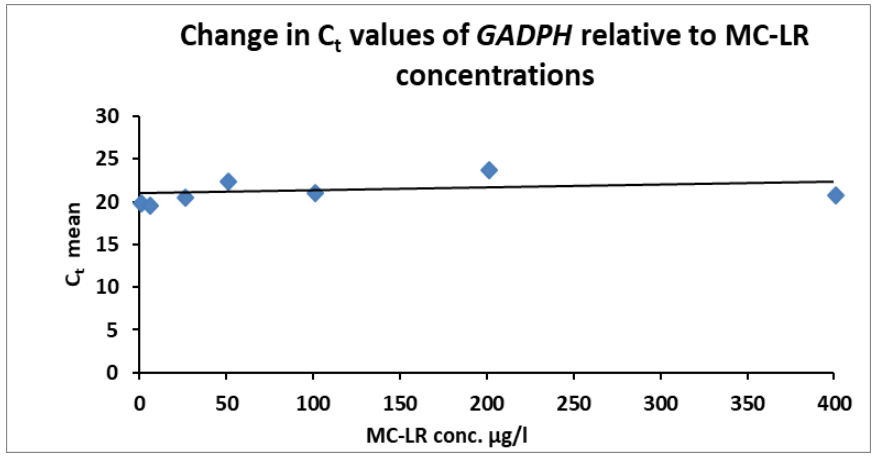
Protein Phosphatase gene expression showed significant responses with MC-LR / *M. aeruginosa* concentration relationship. The initials values of *PPP1ca* with MC-LR showed significantly up-regulated with lower doses, then gradually down-regulation with the higher doses (Figure 2-22 A). However, significantly down-regulation was shown with the different concentrations of *M. aeruginosa* (Figure 2-22 B).



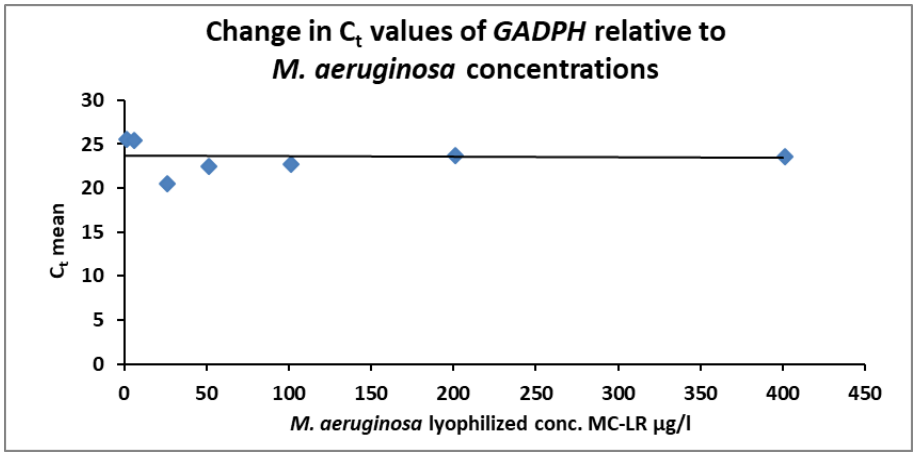
A-1



A-2

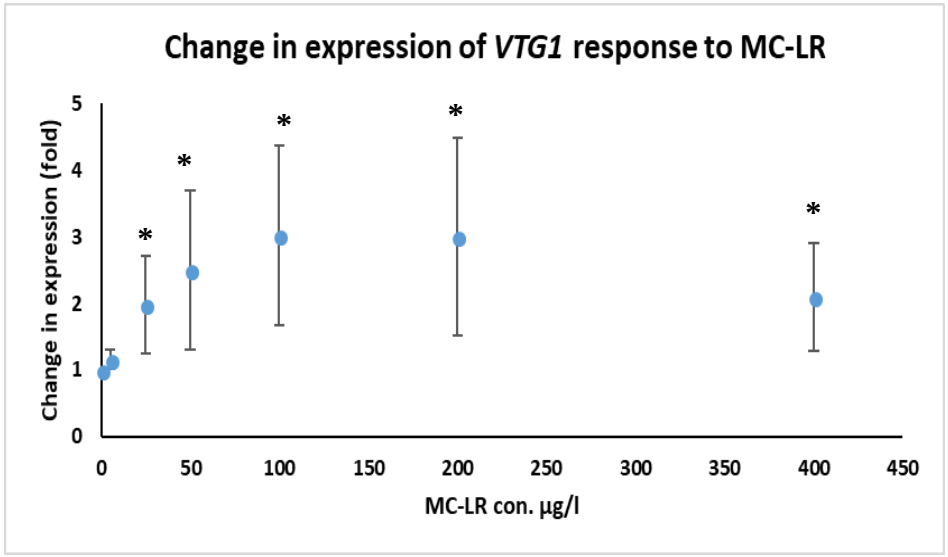


B-1

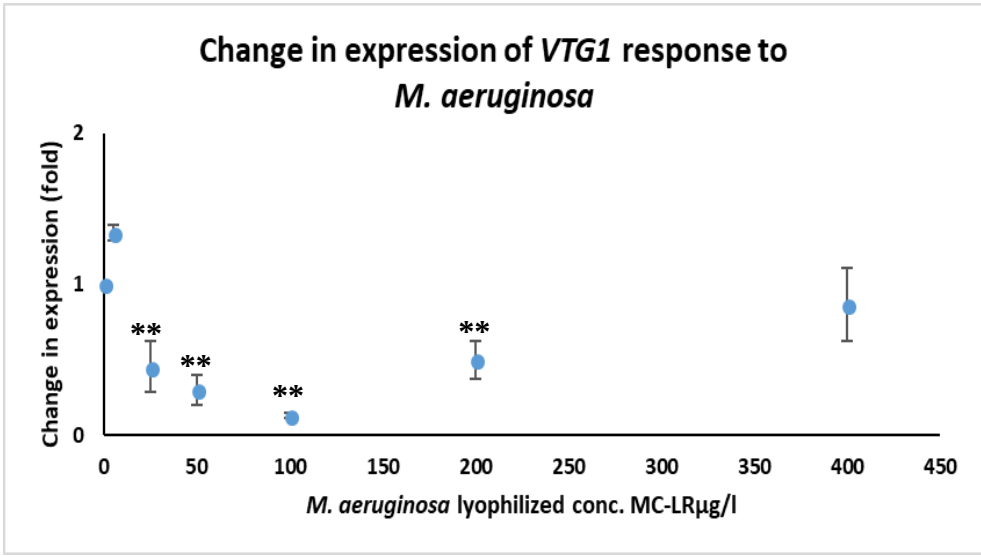


B-2

Figure 2-15: Change in C_t values of reference genes (A 1 & 2) *β-actin and* (B 1 & 2) *GADPH* of zebrafish larvae in response to MC-LR and *M. aeruginosa* doses for 96 hours, (3 replicates for each treatment/ 25 larvae for each replicate).

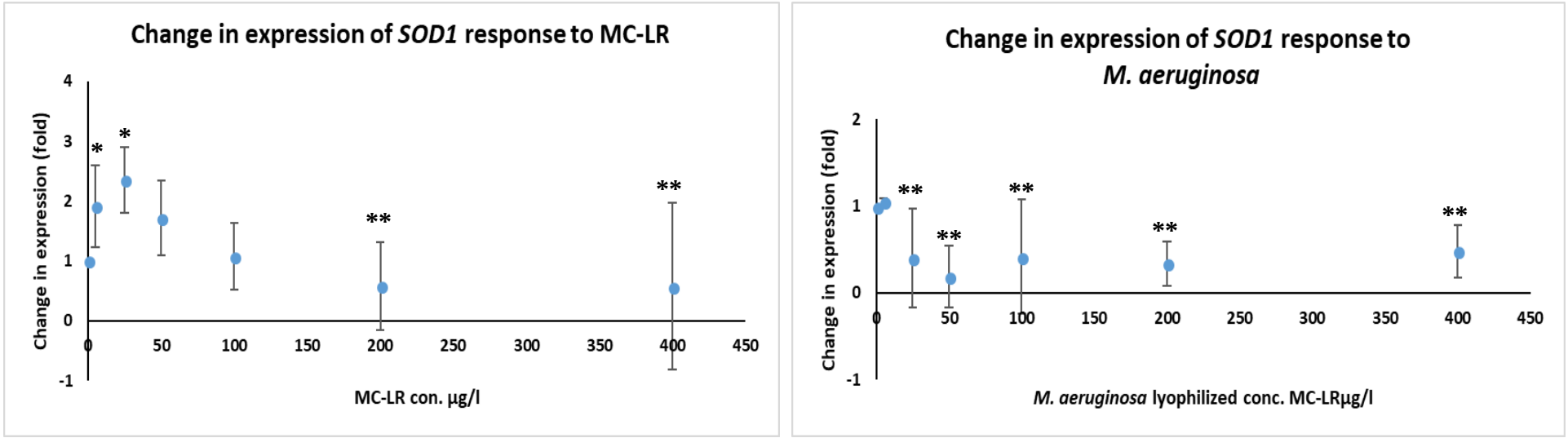


A



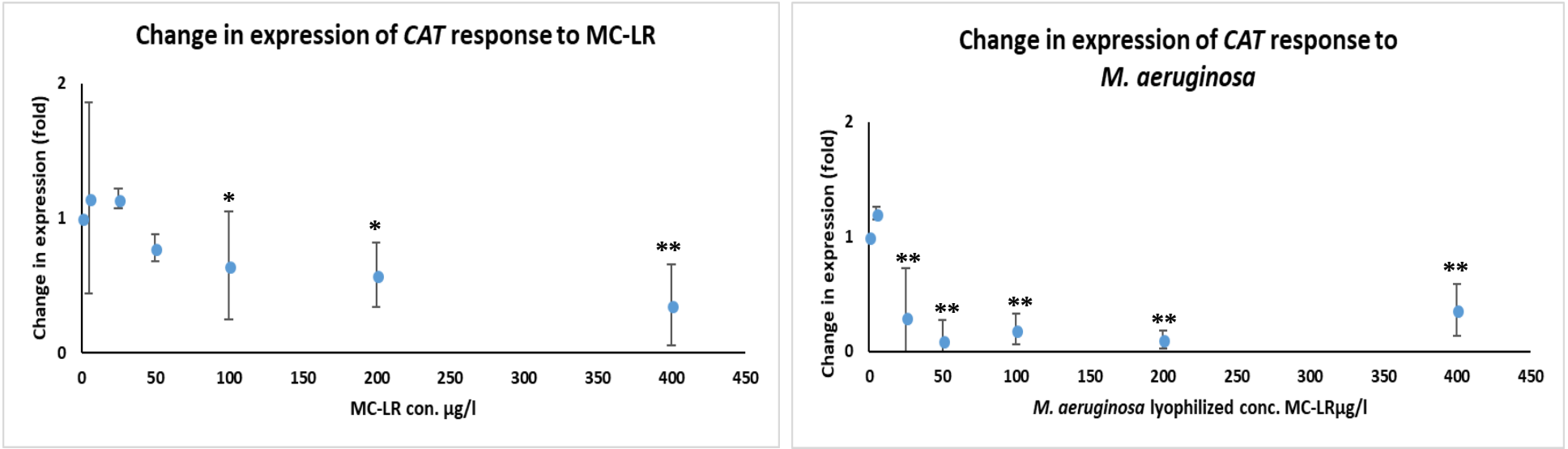
B

Figure 2-16: Change in expression (fold change) of target gene *VTG1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *VTG1* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.



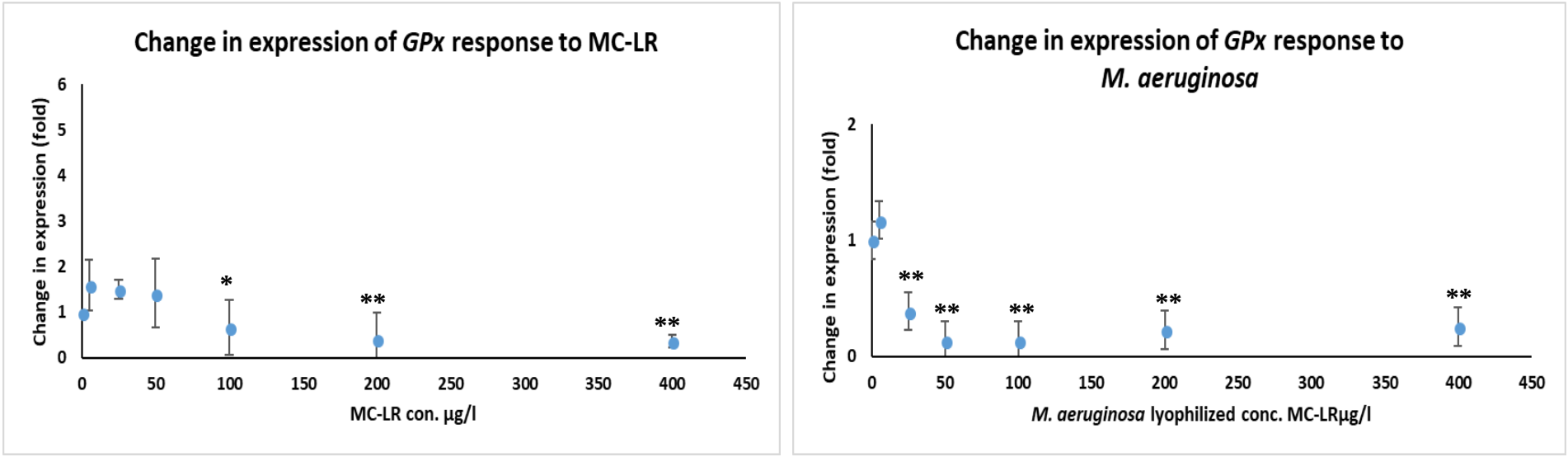
A **B**

Figure 2-17: Change in expression (fold change) of target gene *SOD1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *SOD1* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.



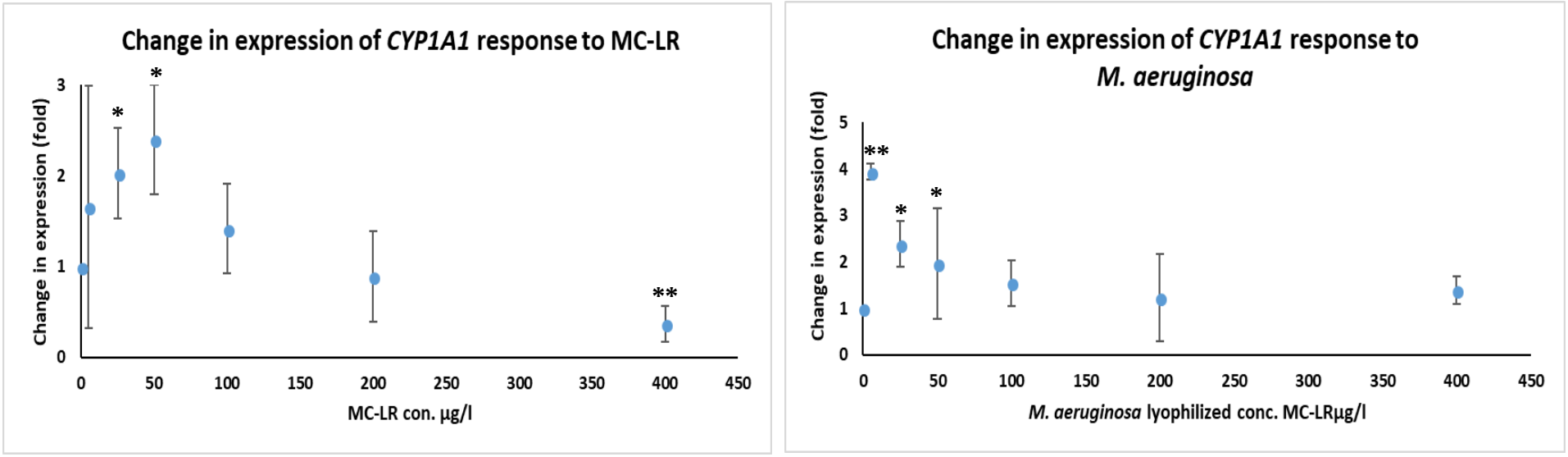
A **B**

Figure 2-18: Change in expression (fold change) of target gene *CAT* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *CAT* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. /3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.



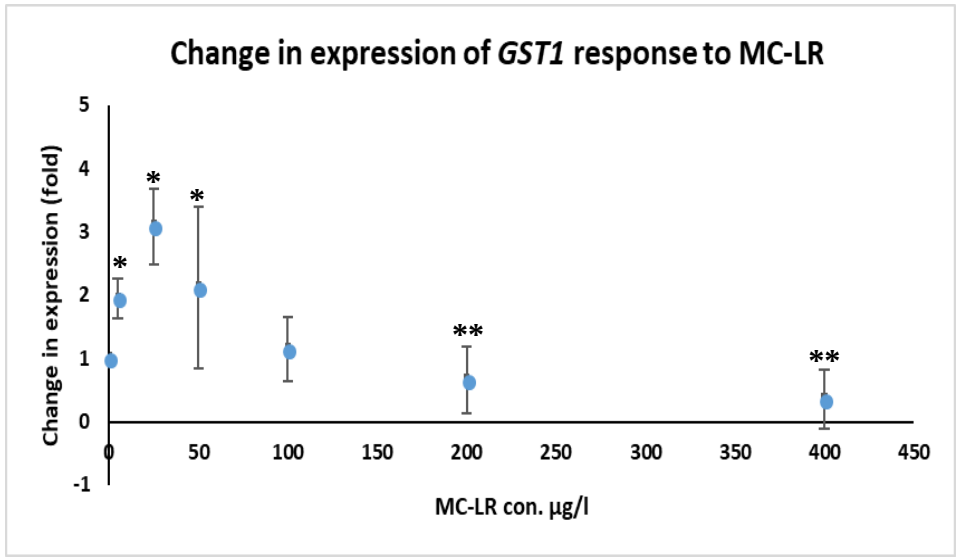
A **B**

Figure 2-19: Change in expression (fold change) of target gene *GPx* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *GPx* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

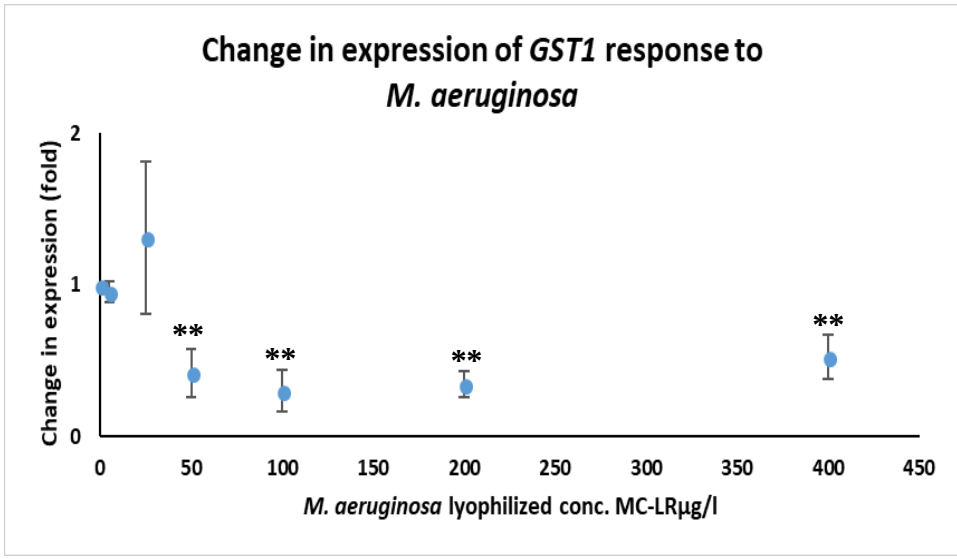


A **B**

Figure 2-20: Change in expression (fold change) of target gene *CYP1A1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *CYP1A1* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. /3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

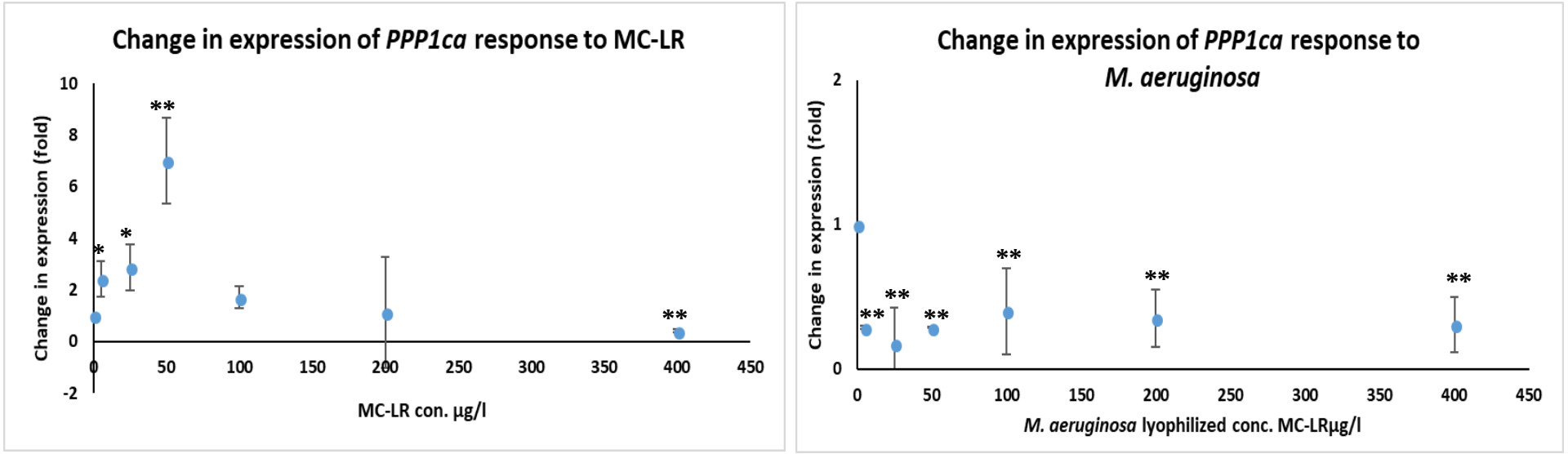


A



B

Figure 2-21: Change in expression (fold change) of target gene *GST1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *GST1* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

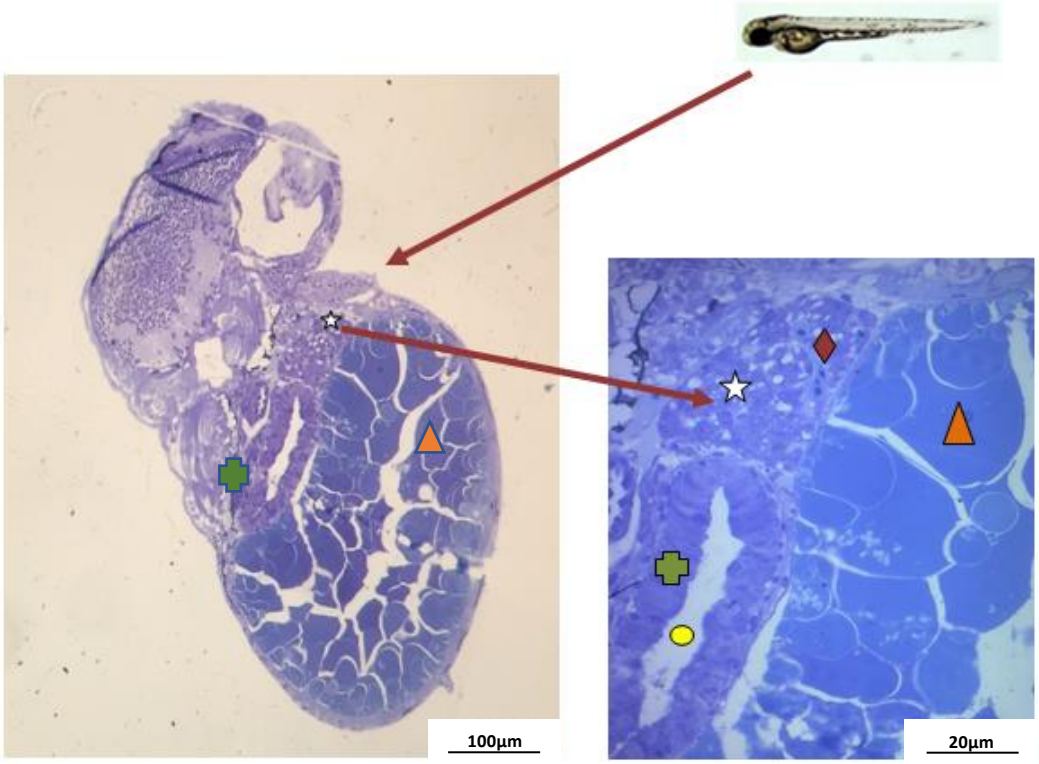


A **B**

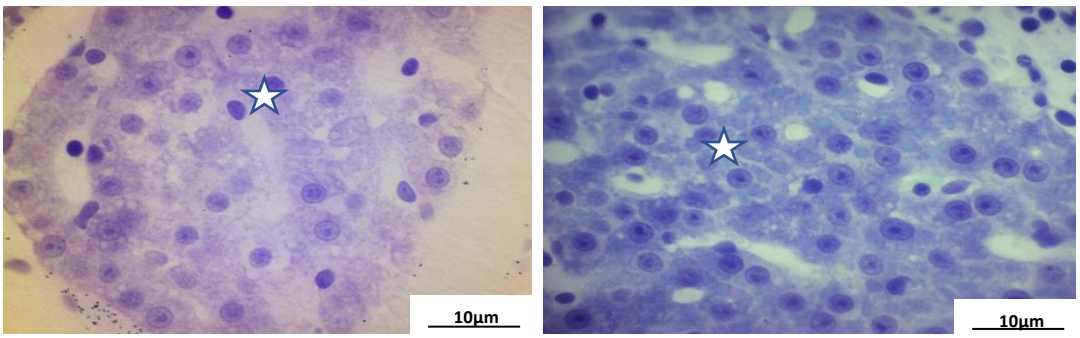
Figure 2-22: Change in expression (fold change) of target gene *PPP1ca* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. Fold changes in expression were calculated by ΔΔCT method with *PPP1ca* expression normalized to zebrafish *GADPH* gene. The results represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

2.4.1.3. Histopathology

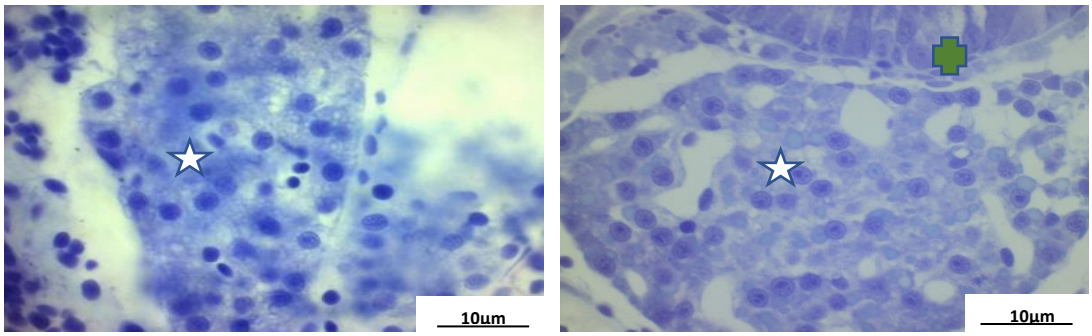
The histopathological results showed that either the MC-LR or *M. aeruginosa* concentration relationship did not show any histopathological changes in the liver or the gut for zebrafish larvae in comparison to the control group (Figures 2-23 A-G). However, 400 µg/L *M. aeruginosa* treatment showed that there was an evacuated appearance and there was more damage to the liver tissue in comparison to the control group.



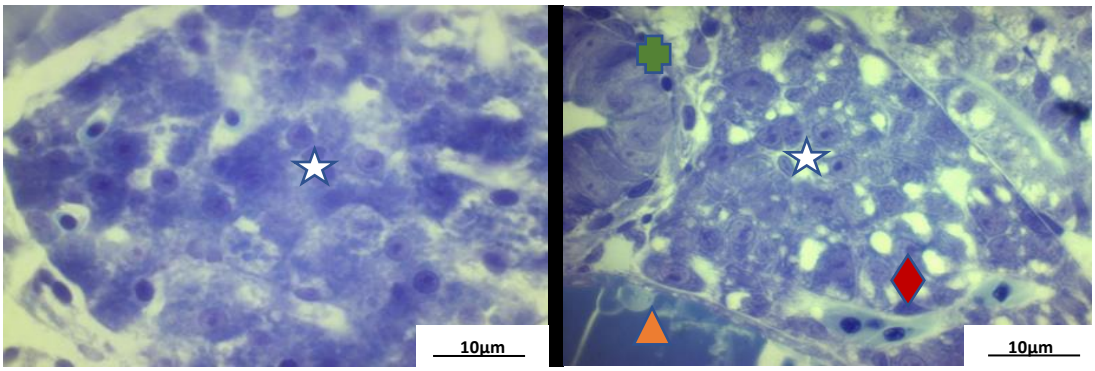
A- General view for one of the treatments



B- Control **C-Control**



D- 50 µg/L MC-LR **E- 50 µg/L *M. auroginosa***



F- 400 µg/L MC-LR **G- 400 µg/L *M. auroginosa***

Figure 2-23: Histopathology sections stained with Toluidine Blue (TB) for zebrafish larvae after plastic histology process. A) 100x general view transverse sections for zebrafish larval and 400x showing the liver (white star), the blood vessel (red diamond), the yolk (white and orange triangle), the intestine (green cross) and the lumen (yellow circle). B-G) 1000x represented liver and gut sections for different concentrations of MC-LR and *M. aeruginosa.*

2.4.2. Experiment 2: MC-LR / *M. aeruginosa* time relationship

2.4.2.1. Gene expression

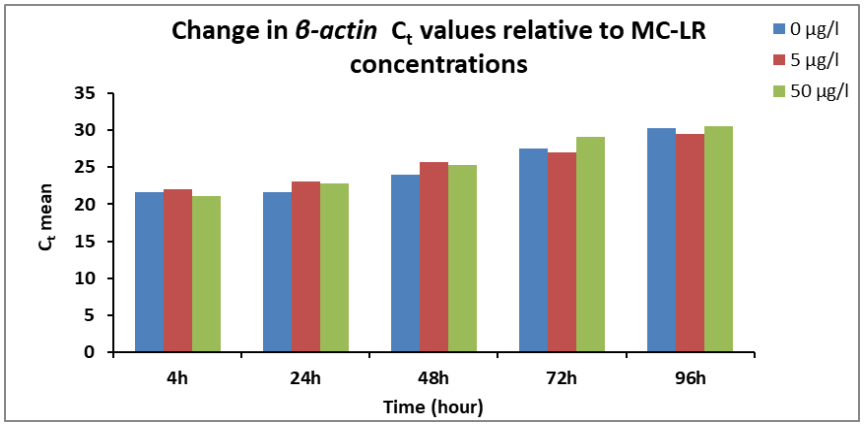
Gene expression profile revealed an overall significant response during the time course after the aqueous exposure to MC-LR / *M. aeruginosa*. The results of the reference genes *β-actin* and *GADPH* were quite interesting. As the results showed that *β-actin is* reducing the time course (increasing the C_t values) (Figure 2-24 A). Additionally, *GADPH* with MC-LR was also a little decreasing through the time, but with *M. aeruginosa GADPH* was nearly stable through the time (Figure 2-24 B). From these results, *GADPH* was used as a reference gene to analyse the Q-PCR data.

In the context of the target genes, *VTG1* showed significant responses with MC-LR / *M. aeruginosa* time relationship. The fish that exposed to MC-LR showed that there was significant induction ($p \leq 0.05$) and ($p \leq 0.001$) of *VTG1* in the early time exposure and then down-regulated by the time. However, *M. aeruginosa* time relation exposure results for *VTG1* showed that there was gradually significant up-regulation starting from 24 hours (Figures 2-25 A&B).

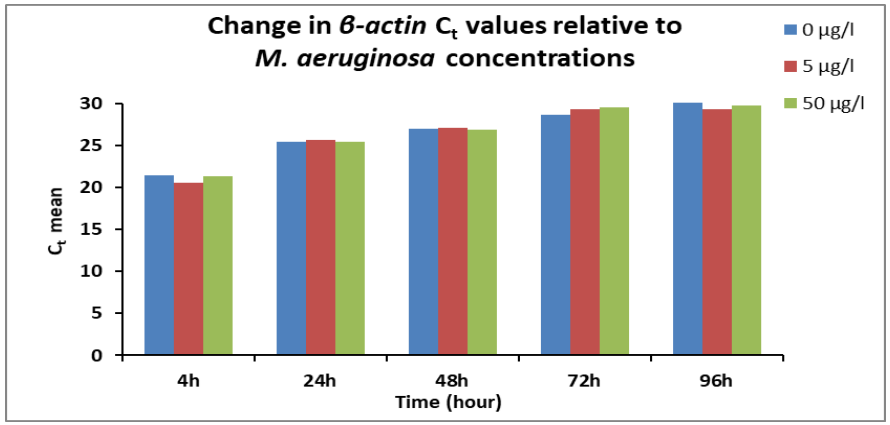
In the context of the target genes, oxidative stress-related genes showed significant responses with MC-LR / *M. aeruginosa* time relationship. Oxidative stress genes (*CAT*, *SOD1* and *GPx*), overall there was significant ($p \leq 0.05$) and ($p \leq 0.001$) up-regulation for MC-LR on early time 24 hours and then gradually down-regulation to no induction at nearly 72 hours and 96 hours. However, *M. aeruginosa* showed no induction during the various times, except at 24 hours there was some an induction for all (*CAT*, *SOD1* and *GPx*) (Figures 2-26 to 2-28).

Biotransformation related genes showed significant responses with MC-LR / *M. aeruginosa* time relationship. The results of time course exposure of MC-LR showed significant induction of *CYP1A1* during the time cause for the higher concentrations starting from 24 hours. Additionally, *GST1* showed significant ($p \leq 0.05$) and ($p \leq 0.001$) induction during the times for the low and the higher concentrations. Also, zebrafish larvae that exposed to *M. aeruginosa* showed significant ($p \leq 0.05$) and ($p \leq 0.001$) induction of *CYP1A1* for nearly the low and the higher concentrations starting from 24 hours, but no induction recorded for *GST1* except slightly induction at 48 hours (Figures 2-29 & 2-30).

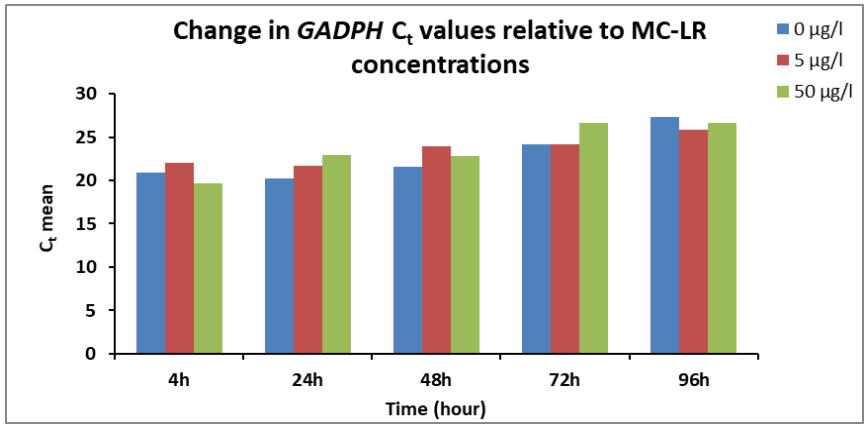
Protein Phosphatase gene showed significant responses with MC-LR / *M. aeruginosa* time relationship. The initials results showed that *PPP1ca* with MC-LR showed significant ($p \leq 0.05$) and ($p \leq 0.001$) induction starting from 48 up to 96 hours, and no induction was showed with *M. aeruginosa* exposure, except significant down-regulation at 96 hours (Figure 2-31).



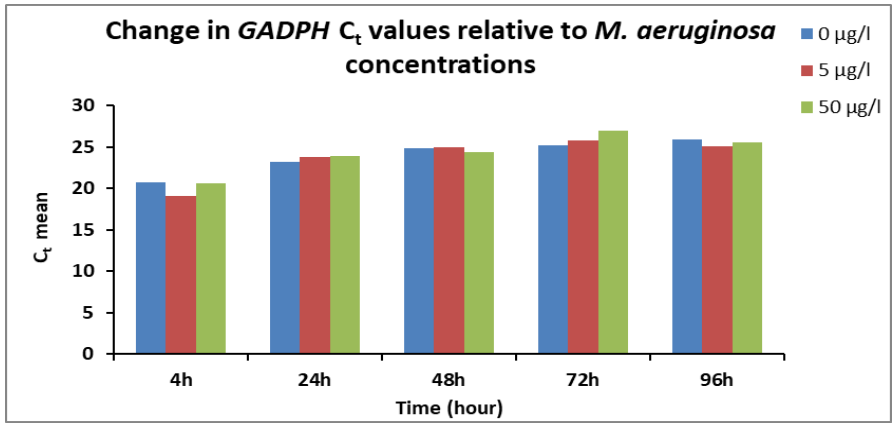
A-1



A-1

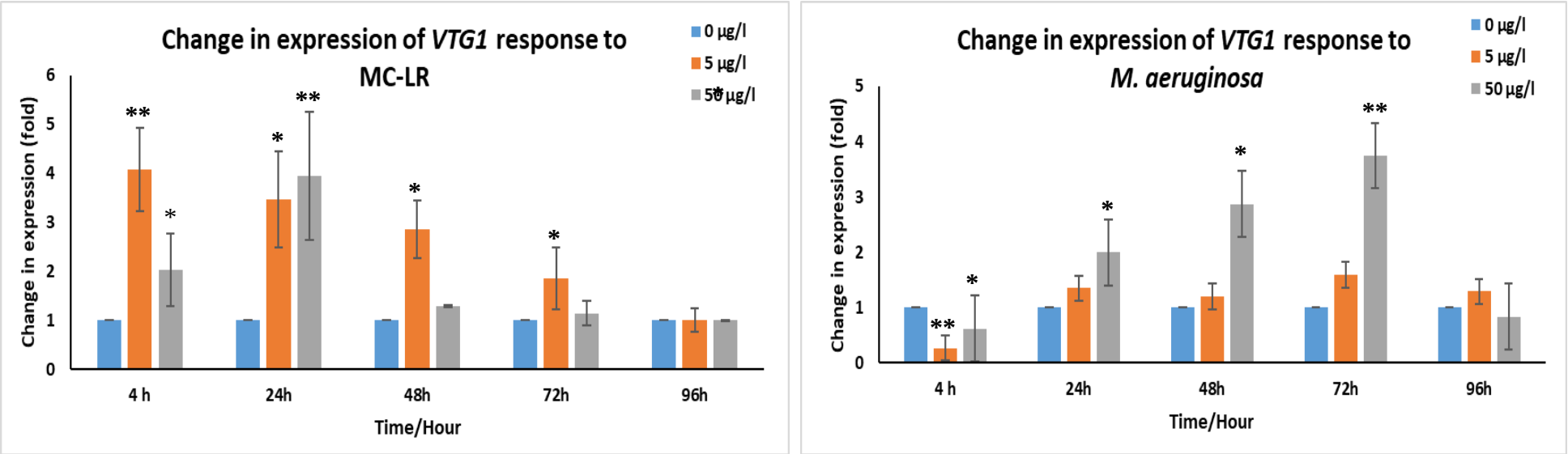


B-2



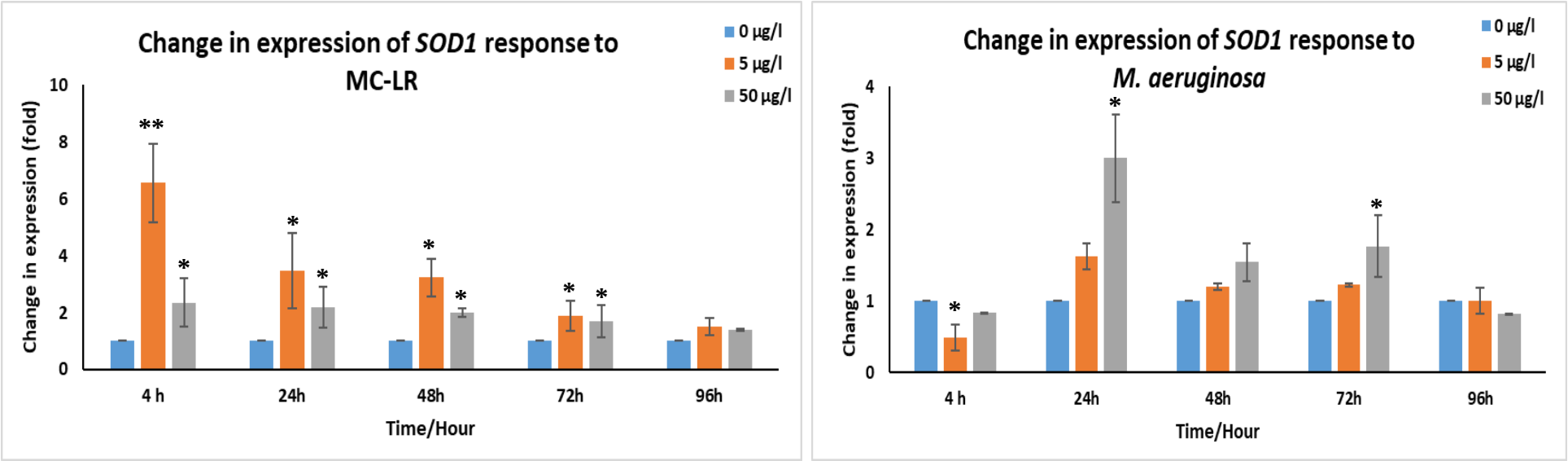
B-2

Figure 2-24: Change in C_t values of reference genes (A 1 & 2) *β-actin and* (B 1 & 2) *GADPH* of zebrafish larvae in response to MC-LR and *M. aeruginosa* doses for 96 hours. The results are showing different times of sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate).



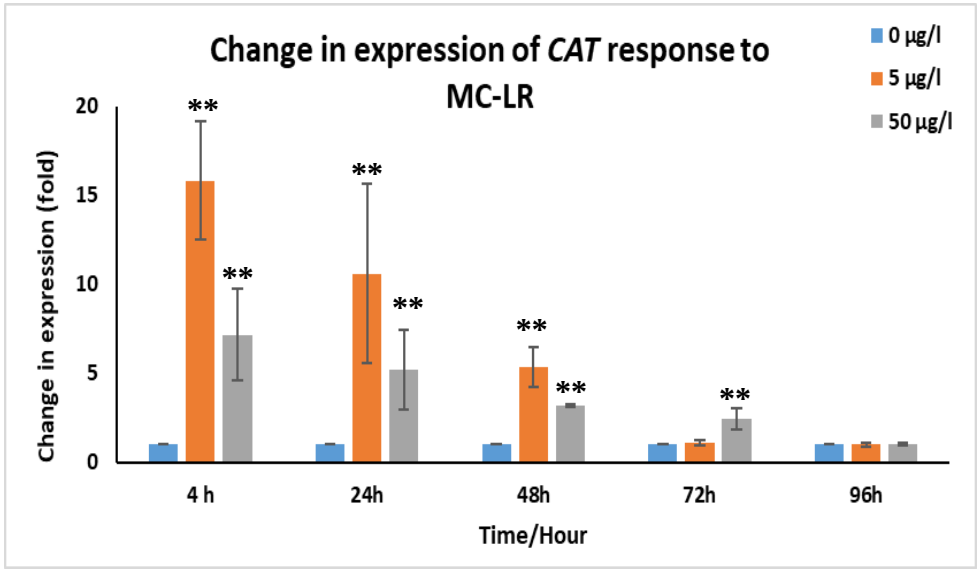
A **B**

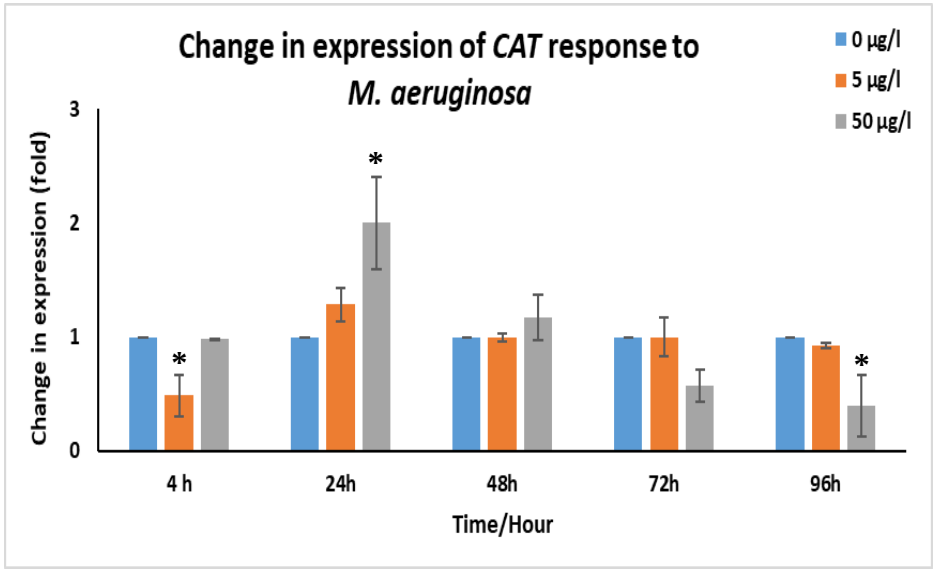
Figure 2-25: Change in expression (fold change) of target gene *VTG1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *VTG1* expression normalized to zebrafish *GADPH* gene.



A **B**

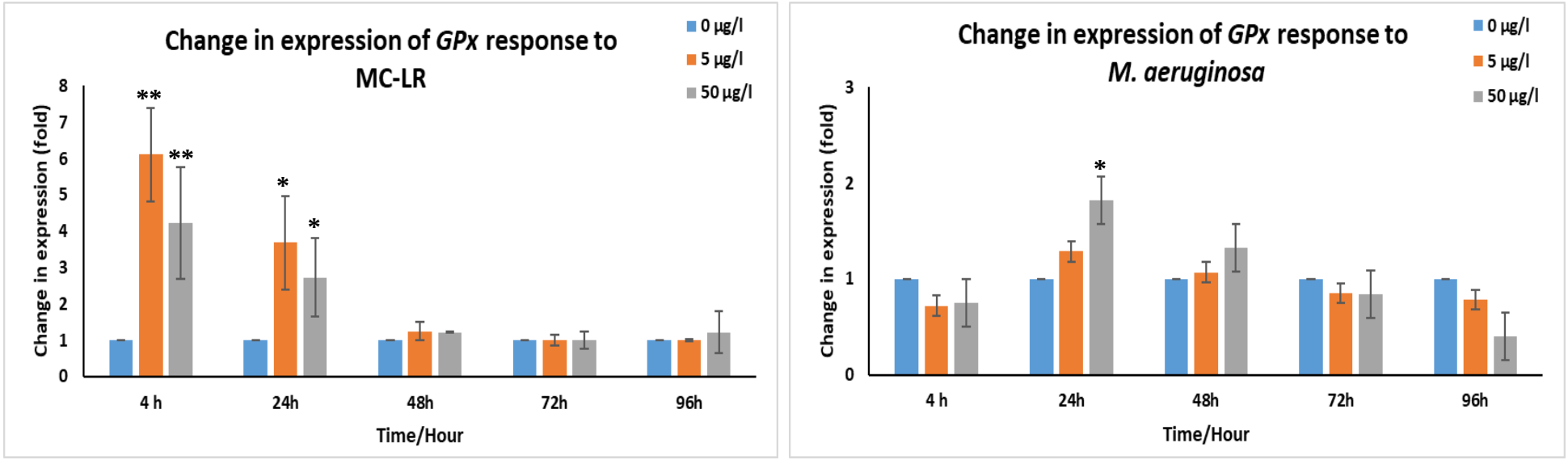
Figure 2-26: Change in expression (fold change) of target gene *SOD1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *SOD1* expression normalized to zebrafish *GADPH* gene.





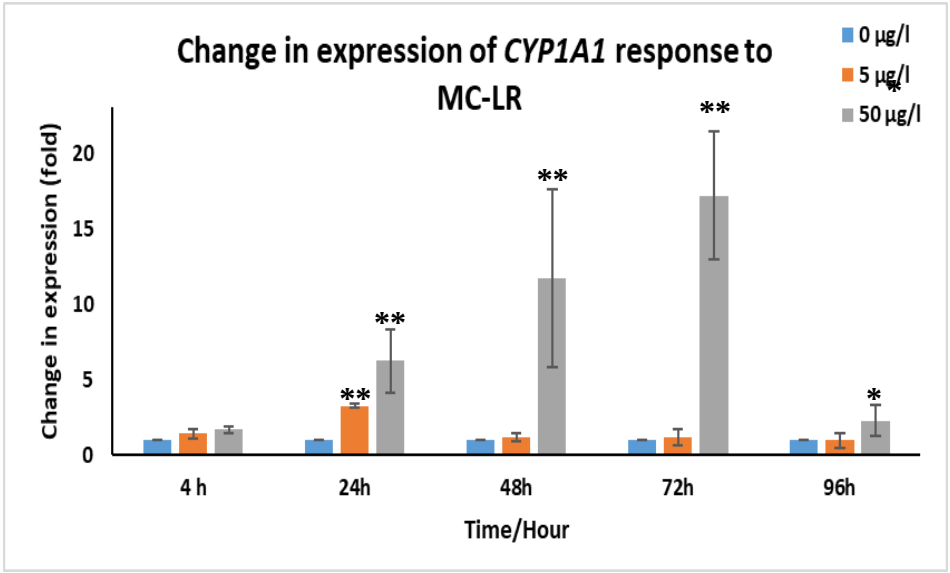
A **B**

Figure 2-27: Change in expression (fold change) of target gene *CAT* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *CAT* expression normalized to zebrafish *GADPH* gene.

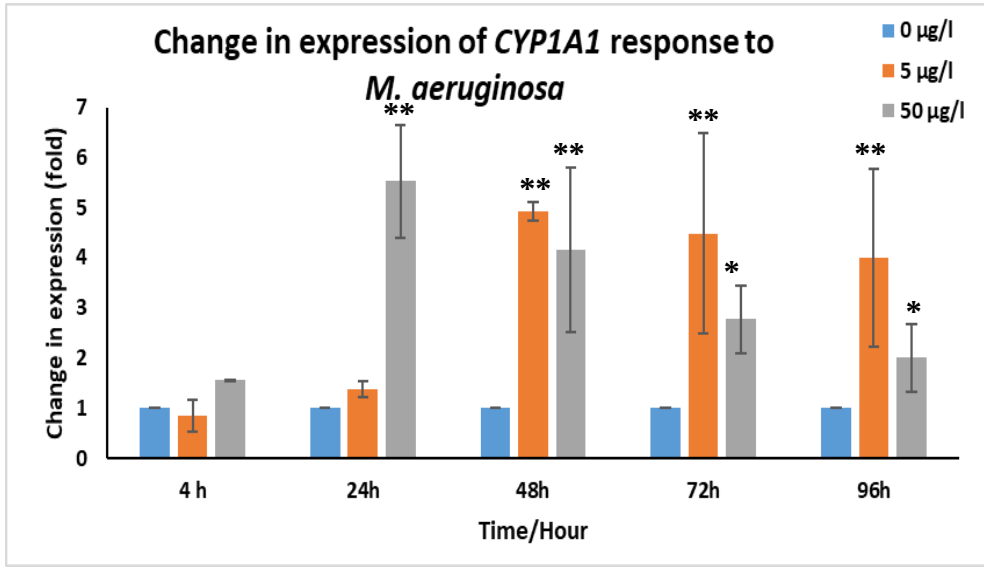


A **B**

Figure 2-28: Change in expression (fold change) of target gene *GPx* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *GPx* expression normalized to zebrafish *GADPH* gene.

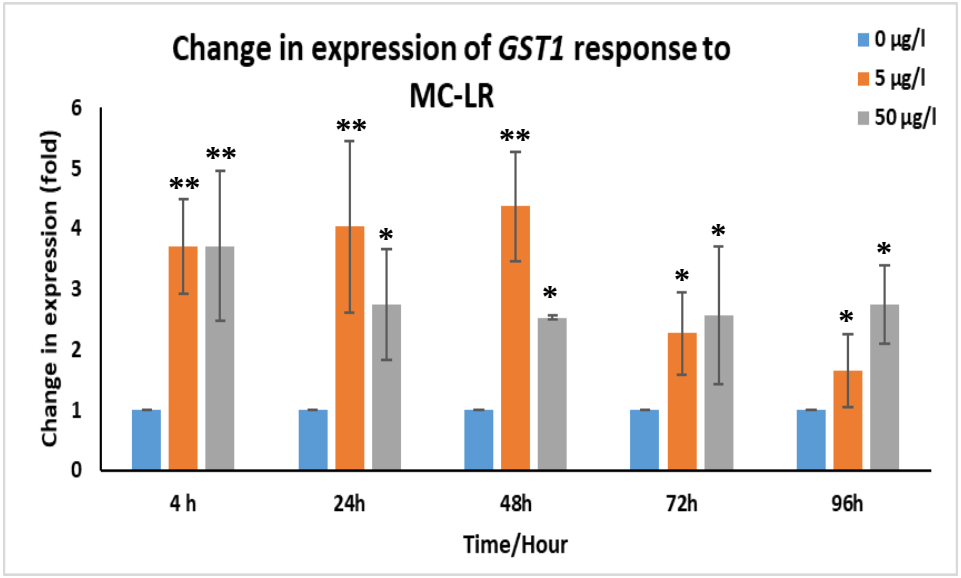


A

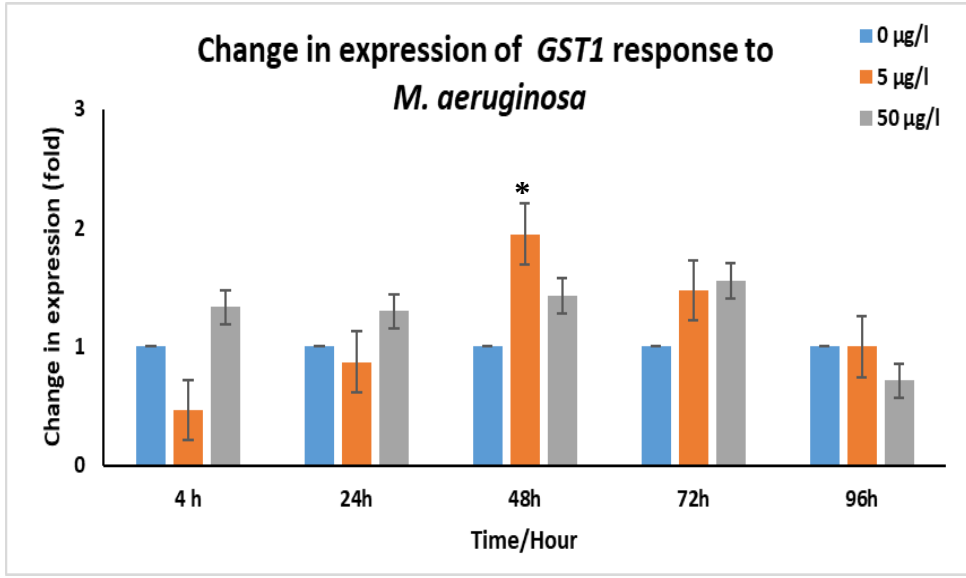


B

Figure 2-29: Change in expression (fold change) of target gene *CYP1A1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *CYP1A1* expression normalized to zebrafish *GADPH* gene.

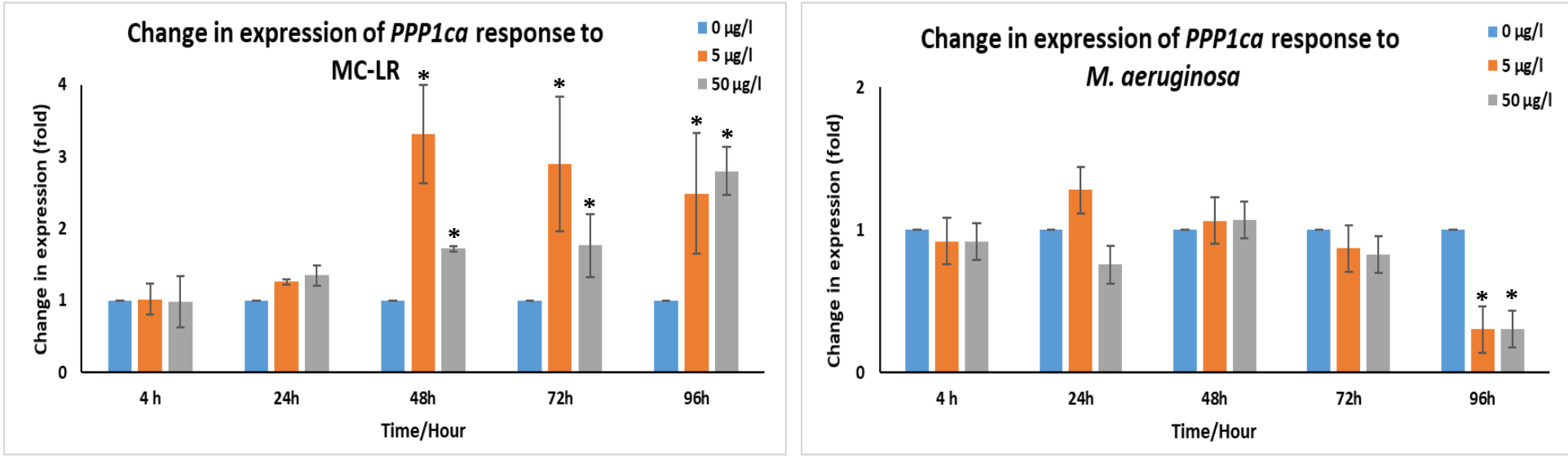


A



B

Figure 2-30: Change in expression (fold change) of target gene *GST1* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. /3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *GST1* expression normalized to zebrafish *GADPH* gene.



A **B**

Figure 2-31: Change in expression (fold change) of target gene *PPP1ca* of zebrafish larvae in response to MC-LR treatments (A) and *Microcystis aeruginosa* treatments (B) for 96 hours. The results showing different times sampling and represented by (mean ± S.E. / 3 replicates for each treatment/ 25 larvae for each replicate), asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group. Fold changes in expression were calculated by ΔΔCT method with *PPP1ca* expression normalized to zebrafish *GADPH* gene.

2.5. Discussion

2.5.1. Experiment 1: MC-LR / *M. aeruginosa* concentration relationship

2.5.1.1. Mortality and deformity

Mortality & deformity results showed lower toxicity of either MC-LR or *M. aeruginosa* than previous reports for zebrafish embryos. A previous study by El Ghazali et al. (2009) showed that after aqueous exposure of zebrafish embryos to *M. aeruginosa* at concentrations of (0, 0.3, 0.1 and 0.03 mg/L) for 24 hours, all embryos died at the highest concentration (El Ghazali et al., 2009). Also, the present results showed that no deformity or abnormal shape was detected except some basal incidental deformity rate, which was 2-3 larvae that had an abnormal shape with a curve in the body in comparison with normal larvae in the control group, confirming the results from the previous study (El Ghazali et al., 2009). The differences between the current results and El Ghazali et al. (2009) that the lyophilized *M. aeruginosa* had a content of 976 g MC-LR/g dry weight, which is higher than the MC-LR concentration in the lyophilized *M. aeruginosa* that was used in this current study. Also, Rogers et al. (2010) had the similar results to the present study, as their selected concentrations 100 and 1000 µg/L of MCLR and *M. aeruginosa* caused no significant mortality (less than 2%) and no observable behavioral changes in larval zebrafish during the 96 hours aqueous exposure.

2.5.1.2. Gene expression

- **Reference gene (Housekeeping gene):**

The housekeeping genes are a good point to start with it. *GADPH* was chosen to be the housekeeping gene to analyse the Q-PCR data. The results indicated that *β-actin* expression was decreasing along with MC-LR concentrations.

- **Target gene (Vitellogenin gene expression):**

Nowadays, *VTG1* expression level is considered an important indicator to detect environmental estrogens (Marin and Matozzo, 2004; Qiao et al., 2013) and reproductive status in fish (Ankley et al., 2009; Park et al., 2010). Zebrafish larvae are considered a perfect tool to assist whether there is an estrogenic effect or not for some substances that would maybe cause *VTG1* induction or reduction (Rogers et al., 2011; Aguirre-Martínez et al., 2017).

The genome of zebrafish contains seven *VTG* genes (*VTG1–7*), named as heterogeneous *VTGs* and the level of the mRNA *VTG1* gene is 100–1000 times higher than any other *VTG* siblings (Wang et al., 2005). The results of the present study showed that mRNA *VTG1* significant induction with MC-LR with the higher doses, which indicated that MC-LR had estrogenic activity. However, the results showed significantly down-regulated mRNA *VTG1* after exposing the larvae to the different concentrations of *M. aeruginosa.* Similarly, Oziol and Bouaïcha (2010) had similar results to the present study, as their result showed a low level of estrogenic response in the human breast carcinoma cell line when cells were exposed to purified MC-LR. Also, their results showed significant induction of estrogen-regulated luciferase gene was observed with MC-LR. However, the results of the present study were different from Rogers et al. (2011) results. Their results showed induction of *VTG1* gene from 19.2-fold to >100-fold when they exposed zebrafish larvae to *M. aeruginosa* at concentrations of 100 and 1000 µg/L for 96 hpf, but no induction was recorded regarding zebrafish larvae exposed to MC-LR with the same concentrations. Therefore, the results of the present study reflected that MC-LR could have a low estrogenic effect, but *M. aeruginosa* did not induce *VTG1* even with the same concentrations of 100 µg MC-LR/L that Rogers et al. (2011) used. So far, the strain of *M. aeruginosa* that used in the present study had nearly the same concentration of MC-LR that Rogers et al. (2011) used. The explanation of these different results may be because of *M. aeruginosa* that Roger et al. (2010) used is releasing estrogenic substances called (aka "phycoestrogens") (Sumpter and Jobling, 1995) and the strain that was used in the present study probably do not release these components, which had a consequence not to induce *VTG1*.

- **Target gene (Oxidative stress-related genes expression):**

MCs can alter the antioxidant system / induce oxidative stress in diverse aquatic species and different organs. Commonly, MCs is considered a phosphatases inhibition besides their effects on alter oxidative stress. MCs exposure may lead to an extreme formation of reactive oxygen species (ROS), which may lead to oxidative damage. Previous reports suggested that there is a connection between cellular hyperphosphorylation state and oxidative stress generation induced by MCs exposure. Additionally, hyperphosphorylated cellular environment induced by MCs exposure may alter antioxidant enzymes, contributing to the generation of oxidative damage (Amado and Monserrat, 2009b). Also, MCs incorporation *per se*, which can be considered the

first event, which activates glutathione depletion and the consequent increase in ROS concentration. Furthermore, MCs uptake has been connected to the production of ROS (Ding et al., 2000, 2001; Li et al., 2003), which leads to an increase in lipid peroxidation (Pinho et al., 2005; Jos et al., 2005; Prieto et al., 2007), DNA damage (Zegura et al., 2003, 2008; Votto et al., 2007), DNA– protein crosslink (Leão et al., 2008), mitochondrial damage (Ding and Ong, 2003) and alteration of the antioxidant defence system (Vinagre et al., 2003; Pinho et al., 2005; Cazenave et al., 2006 a,b; Prieto et al., 2007; Amado et al., 2009b).

To date, no study has done gene expression regarding oxidative stress-related genes on zebrafish larvae after 96 hours exposure to MC-LR or *M. aeruginosa* and the previous studies have focused on the oxidative stress enzymes activity (Wiegand et al., 1999; Cazenave et al., 2006a). Besides that, no direct exposure has been done on zebrafish larvae, as the previous studies have performed the exposure for either MC-LR or *Microcystis* on embryos or adult fish and then testing the effects on larvae after moving them to clean water. Wiegand et al. (1999) investigated the uptake of MC-LR in different life stages of zebrafish by using ^{14}C-labelled MC-LR, which started from first embryonic development up to 5 days old larvae. In their study, they used REKO medium, which was contained the different treatments 0.1, 0.5, 1, 2 and 5 µg MC-LR/L and immersed the embryos in these different doses from ontogenetic development and after hatching (3 and 5 days). Then, they were examining the effects of MC-LR on the enzyme activity for different enzymes on the larvae, after removing them to clean water. Some of their results showed that GPx enzyme increased at 0.5 µg MC-LR/L. In the present study, the concentrations for MC-LR and *M. aeruginosa* were started from 5 µg/L up to 400 µg/L and *GPx* gene expression witnessed slightly increasing in the expression nearly 1.5 fold change corresponding with the lower dose of MC-LR. Similarly to Wiegand et al. (1999), a study by Cazenave et al. (2006b) used different congeners of MCs with zebrafish embryos and also immersed in REKO medium, which contained 25 µg MC-RR/L or 25 µg MC-LF/L for acute exposure for 24 hours. Some of their results were to determine the changes in enzyme activity such as CAT and GPx. Their results showed that there were no changes in the enzyme activity of CAT and GPX. However, in the present study, there was generally significant down-regulation of oxidative stress gene expression for both MC-LR and *M. aeruginosa* for all *CAT, SOD1* and *GPx* especially on the same concentration 25 µg/L that Cazenave et al. (2006b) also used for the congeners that they used. The overall significant down-regulation that occurred in the present study after sub-

lethal aqueous exposure for 96 hours for genes indicated that MC-LR was more toxic from the other MCs congeners that were used in the previous report. Additionally, the larvae are more vulnerable to either MC-LR or *M. aeruginosa* than the embryos. The overall significant down regulations of the oxidative stress-related genes in the present study confirmed the toxicity of MC-LR and *M. aeruginosa* on gene expression level and increasing the oxidative damage and higher ROS. Additionally, hyperphosphorylated cellular environment, which induced by MC-LR exposure may alter antioxidant enzymes, contributing to the generation of oxidative damage. Consequently, this affected the biotransformation related gene expression and reduced them (see next section).

- **Target gene (Biotransformation related genes expression):**

CYP1A1 consider a key for the metabolism or activation of many kinds of procarcinogens, such as polycyclic aromatic hydrocarbons (PAH) (Nebert et al., 2004). A previous report by Hudder et al. (2007) showed that MC-LR exposure made significant induce of *CYP1A2* expression in mice by using the DNA microarray, which suggested that *CYP1A2* may be activated by MC-LR. Another study showed that *M. aeruginosa* decreased the level of CYP450 in mice (Brooks and Codd, 1987). Recent studies reported that MCs induced the change of *CYP3A65* transcription in zebrafish (Li et al., 2013). A recent study by Zhang et al. (2015) provided an insight into the biochemical mechanism related to the toxicity of MCLR in mice. They evaluated the effects of MC-LR on cytochrome P450 isozymes (CYP1A1, CYP2E1 and CYP3A11) at mRNA level, protein content and enzyme activity in the liver of mice that received daily intraperitoneally 2, 4 and 8 μg/kg body weight of MCLR for seven days. Their result showed that MC-LR significantly decreased CYP1A1 and CYP3A11 activities and increased CYP2E1 activity in the liver of mice and MCLR exposure may disrupt the function of CYPs in the liver, which may be partly attributed to the toxicity of MCLR in mice. The inhibition of CYP1A1 and CYP3A11 activities by MCLR exposure suggests that these enzymes may not be involved in the metabolism of MCLR in mice liver. The increase in CYP2E1 activity indicates that CYP2E1 is possibly involved in MCLR metabolism. Also, their results regarding gene expression showed that significantly decreased *CYP1A1* transcription level at 2 μg/kg group, MCLR promoted the expression of *CYP2E1* and MCLR increased *CYP3A11* mRNA level in mice liver after seven days of MCLR exposure. The results of the exposure of MC-LR in the present study partly disagreed

with Zhang et al. (2015) and agreed with Hudder et al. (2007). The results of the present study showed that there was an induction of *CYP1A1* gene expression with lower MC-LR concentrations then significant down-regulated in the higher doses, which suggested that *CYP1A1* gene expression may be activated by MC-LR with the lower doses. Furthermore, *CYP1A1* induced significantly with the lower concentrations of *M. aeruginosa* and gradually down-regulated in the higher concentrations in the present study, which suggested that *CYP1A1* gene expression may also be activated by the lower doses of *M. aeruginosa*. Also, it has been reported that ROS play a role in the decrease of CYP450 activity *in vivo* and *in vitro* (Elbekai and EL-kadi, 2005) and the results of the present study showed that there were an overall down-regulation of the all oxidative stress-related genes, which confirm the toxicity of MC-LR and *M. aeruginosa* on gene expression level and increasing the oxidative damage and higher ROS.

Similar to the results of *CYP1A1* gene, *GST1* gene also showed an induction with the lower doses of MC-LR, however significant down-regulation with increasing concentrations of *M. aeruginosa.* When *CYP1A1* gene expression induced with the lower doses of MC-LR, *GST1* gene expression induced too. *CYP1A1* consider liver phase 1 detoxification and biotransformation, then *GST1* consider liver phase 2 detoxification and biotransformation, as phase 2 take the metabolisms form phase 1 and changing them, so they excreted easily from the body (Zhang et al., 2015). However, the explanation to the down-regulation of *GST1* with *M. aeruginosa* doses is probably because the down-regulation effects were on gene expression level and not on the enzyme activity level to reduce the mRNA in response to increasing the ROS. Confirming the present results by a study by Buryskova et al. (2006) they used the embryos of the African clawed frog to investigate the effects of different cyanobacterial fractions on Frog Embryo Teratogenesis Assay Xenopus (FETAX) and biochemical markers of oxidative stress GPx and biotransformation GST enzymes activity. In their study, they prepared five biomass fractions from different dominant genera (*Microcystis*, *Aphanizomenon*, *Anabaena* and *Planktothrix*) and found that biomarkers especially (GST and GPx) enzymes activity were not affected significantly and in a variable manner, but no effect or clear relation to MC content was noticed.

To date, no previous study has been done any investigation regarding the effects of MC-LR or *M. aeruginosa* on biotransformation related gene expression on zebrafish larvae. Furthermore, the significant down-regulation that the present study showed

regarding the oxidative stress-related genes and *GST1* gene expression confirms the toxicity of MC-LR on the reproductive system. Because of oxidative stress plays a key role in the reproductive toxicity of MC-LR (Hou et al., 2014).

- **Target gene (Protein Phosphatase gene expression):**

Protein phosphatase 2A (PP2A) is a critical regulator of the MC-induced molecular network. It is shown that several molecules and/or signal pathways that are associated with PP2A play important roles in microcystin-induced toxic effects (Liu and Sun, 2015). Previous studies have shown that changes of PP2A activity by MC-LR is considered a primary event during the toxin exposure and could be considered as one of the main mechanisms of MC-LR toxicity (Malbrouck and Kestemont, 2006; Sun et al., 2014; Tzima et al., 2017). PP2A and protein phosphatase 1 (PP1) both belong to the PPP family that contribute most of the serine/threonine phosphatase activity in cells. A well-studied mechanism of toxicity of MC-LR by Strack et al. (2004) showed that MC-LR is a potent inhibitor of PP2A/PP1, which lead to proteins hyperphosphorylation that can be related to the toxicity and the tumor promotion activities. Because of MC-LR higher affinity and its inhibitor ability to PP2A than PP1, the role of PP2A in MC-induced toxicity was well studied. PP2A is a crucial serine/threonine phosphatase, which plays a key role in the regulation of a wide range of cellular processes.

The main forms of PP2A are dimers of catalytic (C) and scaffolding (A) subunits and trimers with an additional variable regulatory B subunit (Strack et al., 2004). MC-LR can bind particularly to the active site of the catalytic subunit of PP2A, which could inhibit its enzymatic activity (Xing et al., 2006), which could mean hyperphosphorylation in the cell and the possibility to increase the level of gene expression of *PPP2caa*. The balance between protein phosphorylation and de-phosphorylation is an important mechanism regulating signal transduction in eukaryotic cells. Its dynamic change almost involved in all the processes from embryonic development to mature adults. Therefore, the reduced PP2A activity caused by MCLR exposure alters a series of key cellular effects, such as cell cycle, cell proliferation, division, signal transduction, and gene expression (Sun et al., 2014).

A study by Tzima et al. (2017) showed that PP2A enzyme activity witnessed 40% reduction in zebrafish larvae that were exposed to 500 µg/L of MC-LR for four days. This reduction in PP2A refers to early effects of MC-LR, as PP2A is more sensitive to MC-LR than PP1 (Tzima et al., 2017). The results of the present study showed significant induction with the lower doses of MC-LR, which possibly mean that reduced PP2A/PP1 activities caused by MCLR and consequently mean that the exposure might alter series of key cellular effects, such as cell cycle, cell proliferation, division, signal transduction, and gene expression (Sun et al., 2014). In addition, this induction is consisted with enhancing enzyme activity at this time point. Also, at 400 µg/L of MC-LR concentration revealed significant down-regulation of *PPP1ca* gene, as both PP1 and PP2a from the same protein phosphatase family, which mean an indication of MC-LR toxicity to inhibit PP1 on the level of gene expression. Furthermore, the significant down-regulation that was shown in the present study with *M. aeruginosa* means that the different concentrations of *M. aeruginosa* could have inhibited *PPP1ca,* as the lyophilized algae have a mix of different toxins, not just MC-LR (Figure 2-8 B).

2.5.1.3. Histopathology

To date, no previous studies have performed any histological study for zebrafish larvae after exposing the larvae for either MC-LR or *M. aeruginosa*. In the present study, the main focus regarding the histological features was on the whole larvae, particularly the liver and the gut. The liver is considered the main target for MC-LR as well as the gut was crucial to make a comparison with the dietary experiment (chapter 3). Additionally, MC-LR is not cell permeant, as it required uptake by the bile acid transport system, which present in the cells lining in the small intestine and in the hepatocytes and (Dawson, 1998). A study by Ito et al. (2000) showed that when MC-LR orally taken, it was mainly absorbed in the small intestine through the portal vein. Besides that, the intestine absorbs MC-LR and the metabolic products of gut microflora and they could be carried from gut to liver and other organs by blood (Goel., 2014).

Because the larvae were exposed for a brief period (sub-lethal exposure for 96 hours), which likely did not make any histopathological effects on the larvae among the different treatments. However, the higher dose of *M. aeruginosa* 400 µg/L showed that the liver tissue had evacuation and tissue damage, which might be due to the effects of the other substances in the *M. aeruginosa*. So far, no previous studies have performed any plastic histology processes for zebrafish larvae to make a comparison with the present study.

2.5.2. Experiment 2: MC-LR / *M. aeruginosa* time relationship

- **Reference gene (Housekeeping gene):**

β-actin expression was decreasing with an exposure time of MC-LR and *M. aeruginosa*, which might mean that *β-actin* is decreasing through the growth line of the larvae. So, *GADPH* was chosen to be the housekeeping gene to analyse the Q-PCR data.

- **Target gene (Vitellogenin gene expression):**

VTG1 showed significant responses with MC-LR and *M. aeruginosa*. So far, no previous study has done any research regarding the effects of MC-LR or *M. aeruginosa* on *VTG1* gene expression on zebrafish larvae during the time course. The results of the present study showed that *VTG1* gene expression had a significant induction in the early time point of exposure to MC-LR then decreased by the time. However, there was gradually increased by the time of exposure to *M. aeruginosa*. These results suggested that MC-LR and *M. aeruginosa* had estrogenic and MC-LR activated mRNA *VTG1* very fast and in the early time of the exposure and the opposite with *M. aeruginosa*, as the activation happened in the late time of the exposure.

- **Target gene (Oxidative stress-related genes):**

Oxidative stress genes (*CAT, SOD1* and *GPx*) showed significant up-regulation on early time point 24 hours and then gradually down-regulation to no induction at nearly 72 hours and 96 hours after exposing the larvae to MC-LR. However, *M. aeruginosa* showed no induction during the various times, except at 24 hours there was some an induction for all (*CAT, SOD1* and *GPx*), which may indicate that *M. aeruginosa* was not altering oxidative stress-related genes during the time of the exposure. No previous record exists to make a comparison with it regarding exposing zebrafish larvae to either MC-LR or *M. aeruginosa* during the time course. But, the results of the oxidative stress-related genes confirmed that oxidative stress-related gene expression was activated by MC-LR in the early time point. Additionally, Amado and Monserrat (2009b) suggested that there was a connection between cellular hyperphosphorylation state and oxidative stress generation induced by MCs exposure, which may alter antioxidant enzymes, contributing to the generation of oxidative damage. Also, MCs uptake has been connected to the production of ROS (Ding et al., 2000, 2001; Li et al., 2003). This connection can lead to an increase

in lipid peroxidation (Pinho et al., 2005; Jos et al., 2005; Prieto et al., 2007), DNA damage (Zegura et al., 2003, 2008; Votto et al., 2007), DNA– protein crosslink (Leão et al., 2008), mitochondrial damage (Ding and Ong, 2003) and alteration of the antioxidant defence system (Vinagre et al., 2003; Pinho et al., 2005; Cazenave et al., 2006 a,b; Prieto et al., 2007; Amado et al., 2009b). Confirming the results of the present study, as *PPP1ca* was induced and activated by MC-LR gradually during the time of the exposure in response to the hyperphosphorylation, which mean it is altering the antioxidant system due the oxidative damage and the production of ROS (Ding et al., 2000, 2001; Li et al., 2003) and we have a nice response was recorded in the present study from all the oxidative stress-related genes with MC-LR exposure from the early time point and on.

- **Target gene (Biotransformation related genes):**

The results of time course exposure of MC-LR showed significant induction of *CYP1A* during the time for the higher concentrations starting from 24 hours. Amazingly, *GST1* showed also significant induction during the times for both the lower and the higher concentrations of MC-LR. However, no induction recorded for *GST1* gene expression except slight induction at 48 hours for the zebrafish larvae that were exposed to *M. aeruginosa*, but very nice significant induction of *CYP1A* for nearly the lower and the higher concentrations starting from 24 hours. No previous report exists regarding zebrafish larvae that expose to either MC-LR or *M. aeruginosa* and their effects on *CYP1A1* gene expression. However, similar results were reported on mice and MC-LR by Hudder et al. (2007), as they showed that MC-LR exposure caused significant induction of *CYP1A2* expression in mice by using the DNA microarray, which suggested that *CYP1A2* may be activated by MC-LR. Also, in another study on mice and MC-LR by Zhang et al. (2015), they provided an insight into the biochemical mechanism related to the toxicity of MCLR in mice. They evaluated the effects of MC-LR on cytochrome P450 isozymes (CYP1A1, CYP2E1 and CYP3A11) at mRNA level, protein content and enzyme activity in the liver of mice that received daily, intraperitoneally, 2, 4 and 8 μg/kg body weight of MCLR for seven days. Their results showed that significantly decreased *CYP1A1* transcription level at 2 μg/kg group and MC-LR promoted the expression of *CYP2E1* and MCLR increased *CYP3A11* mRNA level in mice liver after seven days of MCLR exposure. The results of the exposure of MC-LR in the present study partly disagreed with Zhang et al. (2015) and agreed with Hudder et al. (2007). The results of the present study showed that there was an induction of *CYP1A1* gene

expression during the time course of exposure to MC-LR that suggested *CYP1A1* gene expression activated by MC-LR. Also, it has been reported that ROS play a role in the decrease of CYP450 activity *in vivo* and *in vitro* (Elbekai and EL-kadi, 2005). The present study agreed with (Elbekai and EL-kadi, 2005), as it showed that increased oxidative stress-related genes and the biotransformation related genes by the time course of exposing to MC-LR. That means MC-LR altered them and started to affect them on the gene expression level from the early time point. Also, the results of the first experiment showed nearly the same response from both oxidative stress-related genes and the biotransformation related genes at the same doses and 96 hours. *CYP1A1* witnessed induction with *M. aeruginosa* during the time course that means *M. aeruginosa* altered it from nearly the early time point of exposure.

The present study is the only one study that exists regarding investigate the effects of aqueous exposure of MC-LR or *M. aeruginosa* on *GST1* gene expression level on zebrafish larvae. However, a study by Pavagadhi et al. (2013b) focused on the enzyme activity for GST1 during the 24, 48 and 72 hours for zebrafish embryos after exposing the embryos to MC-LR and MC-RR at different doses 0, 20, 40, 80, 100, 200 and 400 ng/ml. They found that the enzyme activity of GST1 was reduced gradually by time and with increasing the MC-LR concentrations, which might suggest the accumulation of MC-LR and further effects on early developmental stages. The results of the present study showed that *GST1* gene expression was induced significantly at 24, 48 and 72 hours, especially at the lower concentration 5 µg/L, as a response to the decreasing the enzyme level of GST1. Also, both CYP1A1 and GST1 work together and phase1 and phase 2 detoxifications pathways. Also, overall no induction of *GST1* with *M. aeruginosa* which means that *M. aeruginosa* might not alter *GST1* on the early time point.

- **Target gene (Protein Phosphatase gene):**

PPP1ca gene expression showed a significant increase from 48 hours and on after exposure to the MC-LR doses. However, no induction of *PPP1ca* was shown with *M. aeruginosa* exposure. No previous report regarding the effects of MC-LR on *PPP1ca* gene expression during the time course exists. However, the results of the present study indicated that MC-LR is an inhibitor for protein phosphatase 1 and 2 (PP2/PP1), which means hyperphosphorylation (Liu and Sun, 2015) so that *PPP1ca* activated and induced by MC-LR in response to the reduction in protein phosphatase level and the

hyperphosphorylation. Also, MC-LR was more effective on activation *PPP1ca* than *M. aeruginosa*, as *M. aeruginosa* contain other substances, which might reduce the effects of MC-LR. Up to date, no previous studies have used time relation effects of MC-LR and *M. aeruginosa* on larval zebrafish to make the comparison.

Chapter Three

Effects of sub-lethal dietary exposure of *Microcystis aeruginosa* and the toxin microcystin-LR on target-gene expression profiles, histopathology and gut microbiota in adult zebrafish

3.1. Introduction

The blooms of cyanobacteria and its cyanotoxins are globally reported (Palus et al., 2007; Graham et al., 2010; Davis et al., 2012). The cyanotoxins, which represent as a health hazard to humans and animals could be accumulated in aquatic animals and transferred to the human (Chen et al., 2009; Camposand Vasconcelos, 2010).

Microcystin (MC) toxins are monocyclic heptapeptides that are produced by some cyanobacteria, including *Microcystis* spp., *Anabaena* spp. and *Planktothrix* spp. (De Figueiredo et al., 2004). The chemical structure of MCs enables numerous congeners of MCs to be produced by substitution of different amino acids at particular positions within the heptapeptide ring. More than 80 congeners were identified of MCs and the most hepatotoxic that widely studied is microcystin-LR (MC-LR) (Zurawell et al., 2005). At a concentration of 1µg/L, the toxicity of MC-LR can occur depending on the aquatic species, the developmental stage and the exposure route (intraperitoneal injection, feeding or immersion) (Pavagadhi and Balasubramanian, 2013a; Wang et al., 2005; Papadimitriou et al., 2012). According to the review by Malbrouck and Kestemont (2006), when fish were exposed to higher concentrations of MCLR (>1000 µg/L), hepatocytes die through necrosis that can lead to tissue disruption and liver failure.

MC-LR targets liver in particular (Carmichael, 1995), but could also target other organs such as kidney, gills and the gastrointestinal tract (Rabergh et al., 1991; Kotak et al., 1996; Carbis et al., 1997) and the reproductive system in mice (Ding et al., 2006), rat (Li et al., 2008; Xiong et al., 2009) and fish (Baganz et al., 1998). The toxicity of MC-LR could affect the cell function by the loss of structural integrity, causing haemorrhaging, necrosis, subsequently affect growth, stress protein activity and lesion formation (Fujiki and Suganuma, 2009). Similarly, Bu et al. (2006) found that MC-LR caused impairment of cellular function, haemorrhaging and necrosis in the livers of mice, rainbow trout *Oncorhynchus mykiss* (Tencalla et al., 1994) and medaka *Oryzias latipes* (Deng et al., 2010; Mezhoud et al., 2008). According to Carbis et al. (1996a), exposure to MCs caused necrosis of kidney cells and gills of carp *Cyprinus carpio*. Up until now, only limited information is available regarding the effects of chronic dietary either of MC-LR or *M. aeruginosa* exposure on fish (Deng et al., 2010; Acuna et al., 2012). However, no previous record regarding sub-lethal dietary effects of MC-LR / *M. aeruginosa* on adult zebrafish, as the response could be considered an important sight to get a whole picture regarding the effects of MC-LR and *M. aeruginosa* on the histopathological changes that could happen for the liver, gut and kidney.

The toxicity of MC-LR is due to the inhibition of serine/threonine protein phosphatase activity (PP1/PP2A) by binding to these enzymes (Runnegar et al.,1993; Fujiki and Suganuma, 2009). Protein phosphatases regulate the phosphorylation by protein kinases. However, the inhibition by MC-LR of these enzymes could cause hyperphosphorylation of the structural proteins and leads to increase gene expression of unregulated protein kinases (Fujiki and Suganuma, 2009). Also, MC-LR toxicity is also a result of oxidative stress, which in consequence leads to either apoptosis or necrosis (Ding and Ong, 2003; Li et al., 2005, 2007; Morena et al., 2005; Cazenave et al., 2006). The fish that exposed to MCs have developed detoxification mechanisms to resist the toxin risks. The glutathione pathway is considered as an essential biochemical mechanism for the formation of glutathione (GSH) conjugates. GSH could increase the water solubility of MC-LR, in both metabolism and elimination (Pflugmacher et al., 1998; Schmidt et al., 2014; Tzmia et al., 2107). It is vital to understand the mechanisms of *Microcystis* / MC toxicity to determine changes in gene expression profiles for the genes of interest within specific pathways and this research area is a priority for investigation.

Zebrafish (*Danio rerio*) is considered a vital model organism in the ecotoxicology studies (Linney et al., 2004; Rocke et al., 2009). Therefore, many previous studies used zebrafish as a model to study the effects of MCs and *M. aeruginosa* such as Oberemm et al. (1997); Oberemm et al. (1999); Best et al. (2002); Wang et al. (2005); Rogers et al. (2011); Pavagadhi et al. (2012), because its genome has sufficient similarity to the mammalian genes. Additionally, the zebrafish gut microbiota are numerically dominated at all stages of the zebrafish life cycle by members of the bacterial phylum Proteobacteria, with the phyla Firmicutes and Fusobacteria also prevalent during larval and adult stages, respectively (Roeselers et al., 2011).

Numerous previous studies focused on MC-LR toxicity were based on aqueous (Tencalla et al., 1994), one-time force feeding (Tencalla and Dietrich, 1997), short-term dietary exposure bioassays (Juhel et al., 2006) and chronic dietary exposure for around two months (Deng et al., 2010; Acuña et al., 2012). To date, there has been no sub-lethal dietary exposure study determining the potential adverse effects of toxic *Microcystis* blooms or toxin MC-LR on gene expression profile, histopathological changes and gut microbiota community on zebrafish. The objectives of this study were to investigate the effects of dietary exposure to *M. aeruginosa*, MC-LR on target gene expression profiles, liver, gut and kidney histopathology and gut microbiota community in adult zebrafish.

3.2. Materials and Methods

3.2.1. Microcystin-LR stock

Please see chapter two section 2.2.2 for more details.

3.2.2. Culturing, storing and lyophilisation (freeze-drying) *M. aeruginosa*

Please see chapter two sections 2.2.3. and 2.2.4. for more details.

3.2.3. Experimantal diets

The control food was prepared by mixing 10 g fish dry food with warm deionised water (DIW) that was mixed with 2.5 % gelatine. Then, ingredients were mixed until a smooth dough was achieved. The dough was then spread to a thin layer and was pressed with a fine metal mesh to get the required food size (1 mm) suitable to feed adult zebrafish. Finally, the food particles were placed in clean foiled Petri-dishes and labelled and covered to dry overnight at room temperature. The experimental food was prepared the same as the control food with adding the required amount of either MC-LR stock solution concentration 16.67 µg/ml or *M. aeruginosa* stock solution concentration 0.025 µg/µl to achieve the experimental diets, i.e., 5 or 10 µg/gram concentrations. Finally, all the experimental food was prepared at once and the same food was used throughout the experiment.

3.2.4. Experimental design

Adult male and female zebrafish (n=120) age ~ 6 months were obtained from the zebrafish facility at Edinburgh University (Edinburgh, Scotland, UK). Fish were placed into a static aeration system (with one-third water change/day) consisting of fifteen glass aquaria (size 3 L) (8 fish mixed sex/tank) in a triplicate design (3 tanks /treatment, randomly distributed). Fish were held for three months before the beginning of exposure and were fed a commercial dry fish food one time/day and with brine shrimp nauplii (*Artemia* spp.) twice/day, (photoperiod 12 hours light: 12 hours dark). When the dietary exposure was started, fish were fed for 14 days with control food (no MC-LR or *M. aeruginosa*) and the experimental food that contained either MC-LR (5 and 10 µg/kg) or *M. aeruginosa* (5 and 10 µg/kg). Fish were fed twice per day (10:00 am and 16:00 pm), and the total amount of food received per day was 3 % of their body weight (~ fish weight 0.6 gram). Feeding rate was re-calculated daily based on the mean weight of fish sampled

during the experiment and feeding behaviour was monitored during the feeding to determine the food is consuming.

3.2.5. Water change and water quality

Before starting the dietary exposure, the water was changed three times per week for each tank and the water samples were collected to measure water pH. Then, during the exposure, two-thirds of the water was changing twice daily (before feeding) for each tank and the water samples were collected twice daily and measurements were taken of water pH and total ammonia nitrogen. The acceptable levels are (7.0 - 7.5) for pH and (0.0 – 0.5 mg/L) for ammonia. These parameters levels were measured by using specific measurements stripes, which were bought from King British, Lincolnshire /UK.

3.2.6. Sample collection and gross observations

All fish were euthanized with an overdose of tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). The euthanized fish were blotted dry with a paper towel, observed and measured for weight, total length and clinical signs (lesions, hemorrhaging and deformities). The fish were randomly sampled at endpoints 6, 24, 48, 96 and 336 hours for assessment of gene expression (liver samples stored at -80 ˚C). In addition, at 336 hours, the fish were dissected for the whole gut samples, which were then stored at -80 ˚C and male and female from each tank replicate were also sampled for histopathology (whole fish without head and tail in 10% neutral buffered formalin NBF). The survival, growth (wet weight and peduncle length), ingestion of the food and the general behaviour for male and female were monitored and recorded.

3.2.7. Target genes, housekeeping genes and the efficiency

Liver samples were assessed by real-time quantitative polymerase chain reaction (Q-PCR) for the following targets genes: catalase (*CAT*), superoxide dismutase1 (*SOD1*), glutathione peroxidase (*GPx*), glutathione-S-transferase (*GST1*), cytochrome P450 (*CYP1A1*) and protein phosphatase (*PPP1ca*). The reference genes were *β-actin* and glyceraldehyde 3-phosphate dehydrogenase (*GADPH*). *GADPH* was selected for analysing the Q-PCR data. The efficiency of the reference genes and for the target genes were between 91% and 110%. Please, see chapter two, section 2.2.6. (Table 2-1) for more details.

3.2.8. Triazol protocol for extracting RNA and DNase treatment

For this experiment, the method of triazol extraction was used, previously described in Chomczynski et al. (2013). For phase separation, samples were homogenised manually with TRI reagent on ice in the fume hood and incubated at room temperature for ~5 min. Then, 80 µl of chloroform was added and the samples were covered tightly, shake by hand vigorously for 15 seconds and then incubated at room temperature for 2–15 min. After that, the samples were centrifuged at 12000xg for at 4°C 15 min; then three layers were separated and the aqueous phase (upper layer) is the RNA. Next, the aqueous phase (RNA) was transferred to a new tube and mixed with isopropanol. After that RNA was washed with 75% ethanol and then 30 µl of molecular grade water was added and was vortexed until RNA pellet was dissolved. Then, the RNA concentration and quality were checked by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer). Finally, the DNase treatment was done by using the DNase kit form (PrimerDesign, Chandler's Ford, UK) to remove DNA from the extracted RNA according to PrimerDesing manufacturer's protocol.

3.2.9. Reverse transcription

After RNA extraction samples were diluted to 100 ng/µl of total RNA, and 800 ng for each sample was used to synthesise complementary DNA (cDNA) proceeding with manufacturer's protocol steps (ImProm-II™ Reverse Transcription System; Promega), with deoxynucleotide mix and hexanucleotide primers (Sigma-Aldrich). In these conditions cDNA was synthesised: annealing at 25 ˚C, extending at 42 ˚C and heat-inactivating transcriptase at 70 ˚C (GeneAmp PCR System, 9700; Applied Biosystems). Finally, cDNA was stored at −20 ˚C until quantitative reverse transcriptase–PCR (qRT–PCR) gene expression analysis.

3.2.10. Quantitative reverse transcriptase–PCR (qPCR)

Please see chapter 2 section 2.4.1.2. for more details.

3.2.11. Histopathology

The whole fishes (males and females) for the different treatments were kept in 10% Neutral Buffered Formalin (NBF) after removing the head and the tail. After leaving the fish in NBF five days, then the fish samples were dehydrated in a graded ethanol series, immersed in xylol and embedded in paraffin wax. For each tissue block, serial sections (7-9 mm thick) were cut and stained with Hematoxylin and Eosin (H&E). The fish samples were examined for a variety of histopathological features and hepatocytes nucleus's sizes. The hepatocytes nucleus size was measured by using ImageJ software.

3.2.12. Gut microbiota

After the dissection, the whole zebrafish gut samples were sampled and placed in liquid nitrogen and were stored at –80 ˚C. Then, DNA extraction was done by using (Qiagen kit). Then, the phenol-chloroform was used to eliminate PCR inhibitors (exonuclease). Next, the PCR was run for the samples to amplify 16S rRNA gene fragments and then the digestion stage for the PCR products was run by using Exonuclease kit. Finally, the gut samples were ready for microbiota assessment through using Terminal Restriction Fragment Length Polymorphism (t-RFLP). The t-RFLP was done by submitting the samples for fragment analysis at Genewiz, (USA). Then, after receiving the data from Genewis, the fragment data preparation was done by using GeneMarker software (www.softgenetics.com/GeneMarker.php) and the analysis was done by using multivariate ecological statistical package PRIMER6 software (www.primer-e.com).

Currently, culturing microbiological methods reflect only 1% of total microbial diversity, therefore it cannot be adopted for microbial community assessment. So, several molecular techniques for evaluating total microbial communities have been developed. So far, the majority of these techniques use PCR to amplify genes of interest directly from environmental samples without a culture bias (Kozdroj and Elsas, 2001; Ranjard et al., 2000; Tiedje et al., 1999). Furthermore, the existing PCR-based methods include Amplified Ribosomal DNA Restriction Analysis (ARDRA), Single-Stranded Conformation Polymorphism analysis (SSCP), Thermal and Denaturing Gradient Gel Electrophoresis (TGGE and DGGE), Amplified Length Heterogeneity analysis (ALH) and Terminal Restriction Fragment (T-RF) patterns or profiles (also known as T-RFLP analysis). So far, T-RFLP is considered a PCR-based tool for studying microbial community structure and dynamics (Figure 3-1).

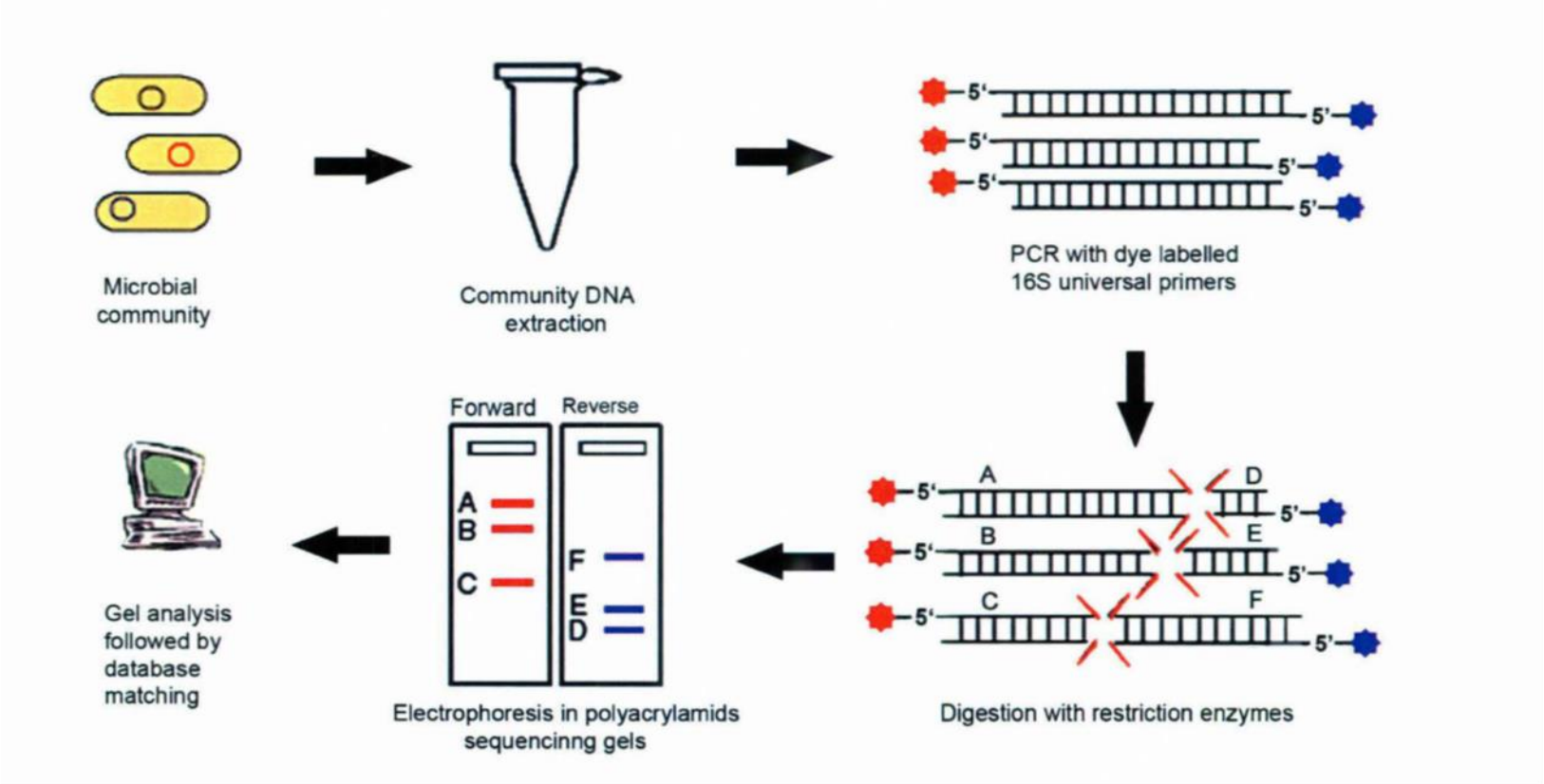


Figure 3-1: Tern1inal restriction fragment patterns are generated and analysed in a series of steps that combine DNA extraction, PCR, restriction enzyme digestion, gel electrophoresis and data analysis. DNA extracted from a sample is subjected to PCR using primers homologous to conserved regions in a target gene. Both the primers (forward and reverse) are labeled on the 5'-ends, usually with two different fluorescent molecules. The amplified DNA fragments (amplicons) are then digested with a restriction enzyme, usually one with a tetranucleotide recognition sequence. The digested amplicons are subjected to electrophoresis in either a polyacrylamide gel or a capillary gel electrophoresis apparatus coupled to a DNA sequencer with a fluorescence detector so that only the fluorescently labeled terminal restriction fragments (T-RFs) are visualized. Finally, individual T-RF peaks in a pattern can be identified by comparison to a clone library or by predictions from an existing database of sequences. This figure with the legend was adopted from (Frostegard et al., 1999).

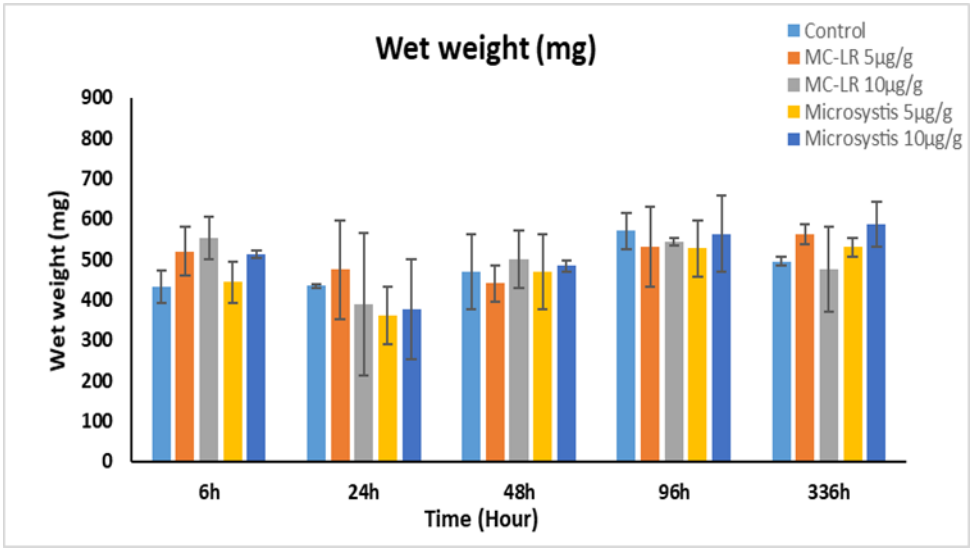
3.2.13. Statistical analysis

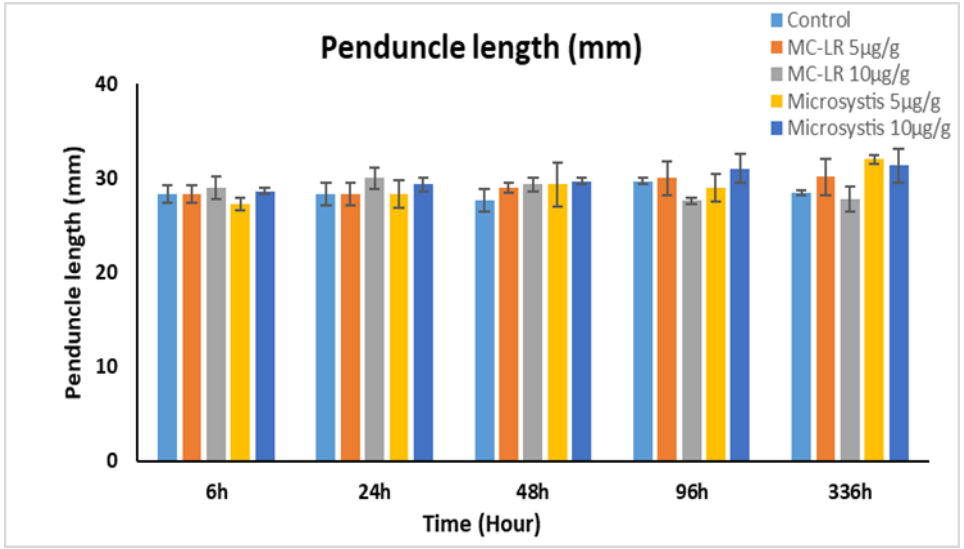
All data except the histology results were statistically analysed by using R-software (R Core Team, 2017). Bartlett homogeneity of variance test was used to test the normal distribution of data. Then, one-way analysis of variance (ANOVA) followed by Tukey's multiple mean comparison tests was used to test differences among dietary treatments in body weight, length, hepatocytes nucleus size and gene expression. The p values ($p \leq 0.05$) and ($p \leq 0.001$) were significant. Data are presented as (means ± standard error). Primer6 software - ANOSIM was used to statistically analyse the differences in bacterial community structure among different treatments.

3.3. Results

3.3.1. Gross observations

Dietary exposure of *M. aeruginosa* / MC-LR did not reveal any significant effects on gross zebrafish observations. All the fish ingested the food and no mortality was observed. In addition, there were no effects in fish behaviour and there were not any significant effects regarding the treatments or time on the wet weight (mg) and peduncle length (mm) at $P \leq 0.05$ (Figures 3-2 A & B).





A **B**

Figure 3-2: (A) the wet weight (mg) and (B) the penduncle length (mm) of adult zebrafish after dietary exposure to different concentrations of MC-LR and *M. aeruginosa* during the time course, 3 replicates for each treatment / one fish form each replicate at 6, 24, 48, 96 hours and 4 fish at 336 hours. The results were represented by (mean ± S.E).

3.3.2. Gene expression

Dietary exposure of *Microcystis aeruginosa* and MC-LR showed an overall significant effect on gene expression profiles for the target genes. *CYP1A1* was induced significantly at $P \leq 0.001$ or $P \leq 0.05$ in the early time's points of exposure within the different treatments and later at 336 hours, especially with MC-LR treatments, but down-regulation at 96 hours (Figure 3-4). *CAT* showed no significant effects during the exposure time except significant response at 24 and 48 hours (Figure 3-3). Furthermore, *GPx, GST1* and *SOD1* revealed significant effects among different treatments at $P \leq 0.001$ or $P \leq 0.05$ in relation to the time (336 hours) with significant effects with some treatments at early time exposure (Figures 3-5 to 3-7). The expression of *PPP1ca* appeared to be affected overall by the different treatments during the time course (Figure 3-8). There was a slight increase with MC-LR lower dose at early time points and then expression was down-regulated, whereas there was an overall down-regulated with the higher dose of MC-LR. Additionally, the induction of *PPP1ca* happened with the lower doses of *M. aeruginosa* at 24 and 48 hours and later at 336 hours. However, there was an overall down-regulation with the higher dose of *M. aeruginosa*.

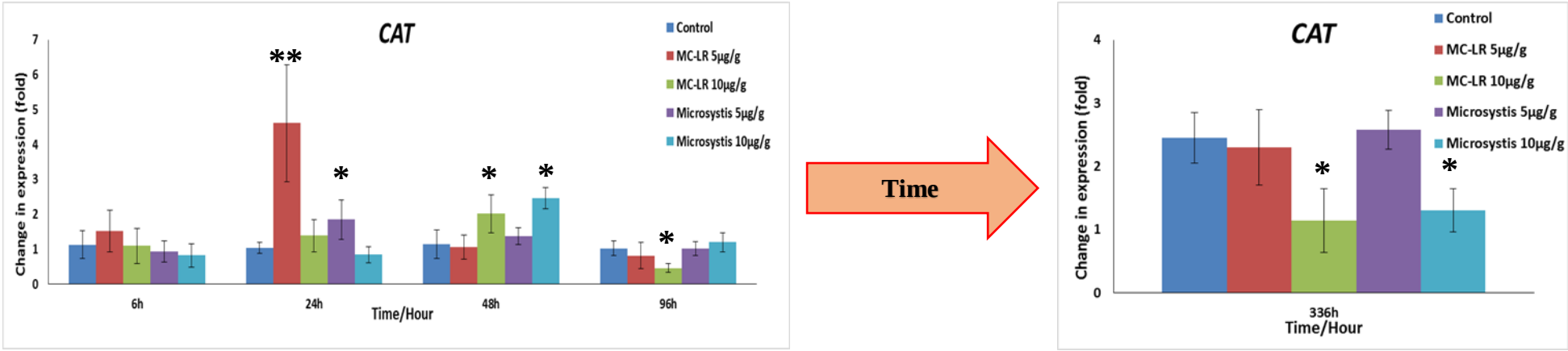


Figure 3-3: Change in gene expression of *CAT* (fold change) of liver samples from adult zebrafish exposed to MC-LR and /or *M. aeruginosa* for 336 hours (mean ± S.E. / n = 8 / 2 fish from each replicate for each time point). Asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

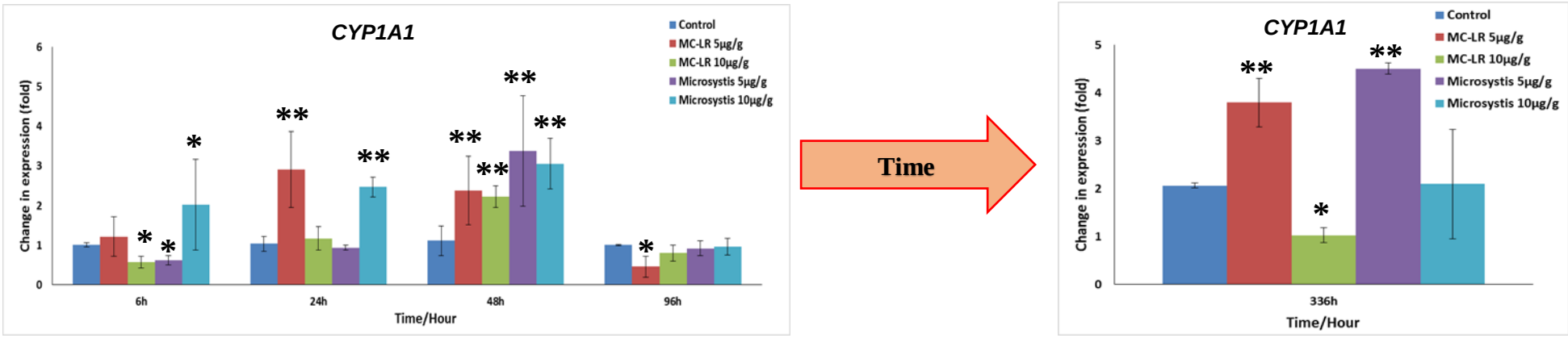


Figure 3-4: Change in gene expression of *CYP1A1* (fold change) of liver samples from adult zebrafish exposed to MC-LR and /or *M. aeruginosa* for 336 hours (mean ± S.E. / n = 8 / 2 fish from each replicate for each time point). Asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

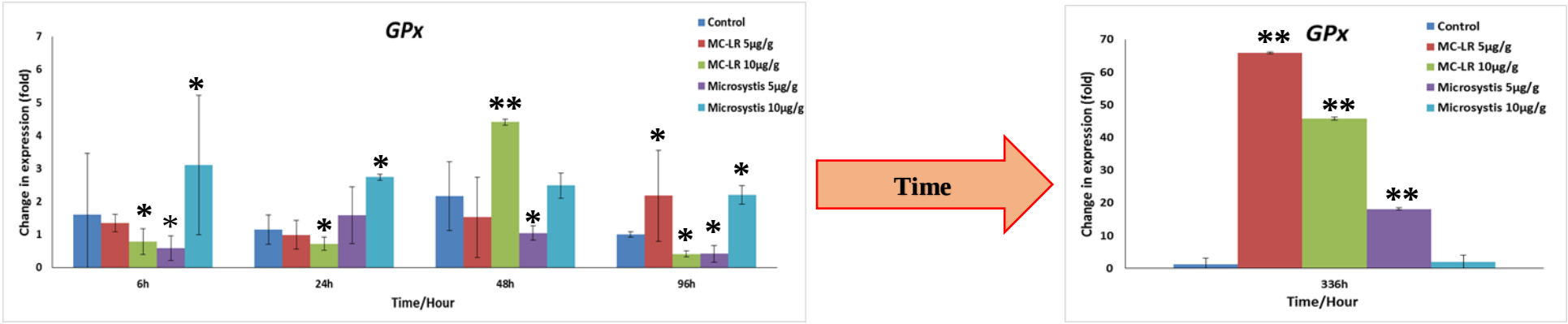


Figure 3-5: Change in gene expression of *GPx* (fold change) of liver samples from adult zebrafish exposed to MC-LR and /or *M. aeruginosa* for 336 hours (mean ± S.E. / n = 8 / 2 fish from each replicate for each time point). Asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

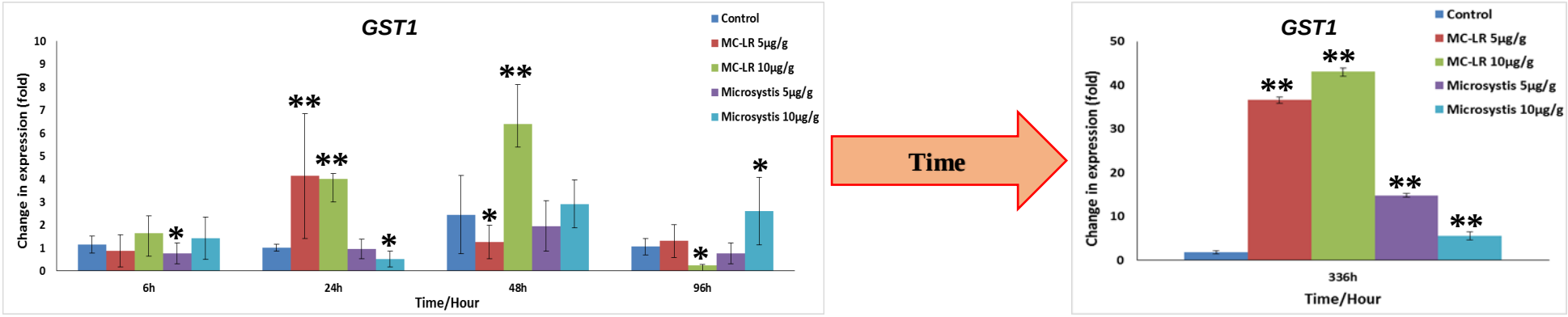


Figure 3-6: Change in gene expression of *GST1* (fold change) of liver samples from adult zebrafish exposed to MC-LR and /or *M. aeruginosa* for 336 hours (mean ± S.E. / n = 8 / 2 fish from each replicate for each time point). Asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

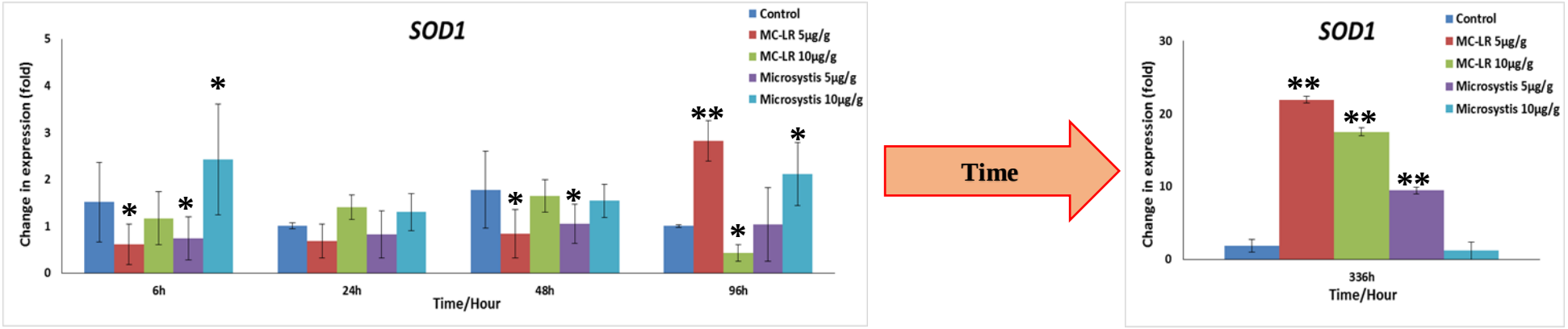


Figure 3-7: Change in gene expression of *SOD1* (fold change) of liver samples from adult zebrafish exposed to MC-LR and /or *M. aeruginosa* for 336 hours (mean ± S.E. / n = 8 / 2 fish from each replicate for each time point). Asterisk (**) and (*) indicate significant differences at $P \leq 0.001$ and $P \leq 0.05$ respectively among different treated groups and the control group.

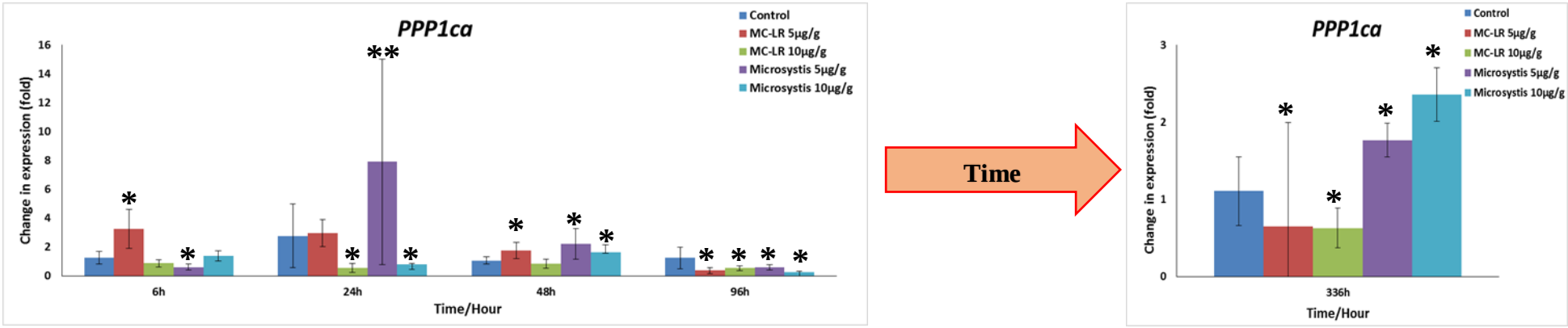


Figure 3-8: Change in gene expression of *PPP1ca* (fold change) of liver samples from adult zebrafish exposed to MC-LR and /or *M. aeruginosa* for 336 hours (mean ± S.E. / n = 8 / 2 fish from each replicate for each time point). Asterisk (**) and (*) indicate significant differences at P ≤ 0.001 and P≤0.05 respectively among different treated groups and the control group.

3.3.3. Histopathology

Dietary exposure of *M. aeruginosa* / MC-LR caused some histopathological changes in some of the zebrafish organs. The histopathology investigation indicates minor lesions were observed in the liver tissue (Figure 3-9 A). Also, the gut samples revealed no histopathological changes with either MC-LR or *M. aeruginosa* treatments (Figure 3-9 B). However, the trunk kidney showed indications of necrosis (karyorrhexis) in fish exposed to 5 & 10 µg/g MC-LR (Figure 3-9 D). Additionally, karyorrhexis, hypertrophy and hyperplasia were observed in fish exposed to 5 & 10 µg/g *M. aeruginosa* (Figures 3-9 E&F) in compared to controls (Figure 3-9 C). Finally, the liver samples showed no significant effects on the hepatocytes nucleuses size (Figure 3-9 G).

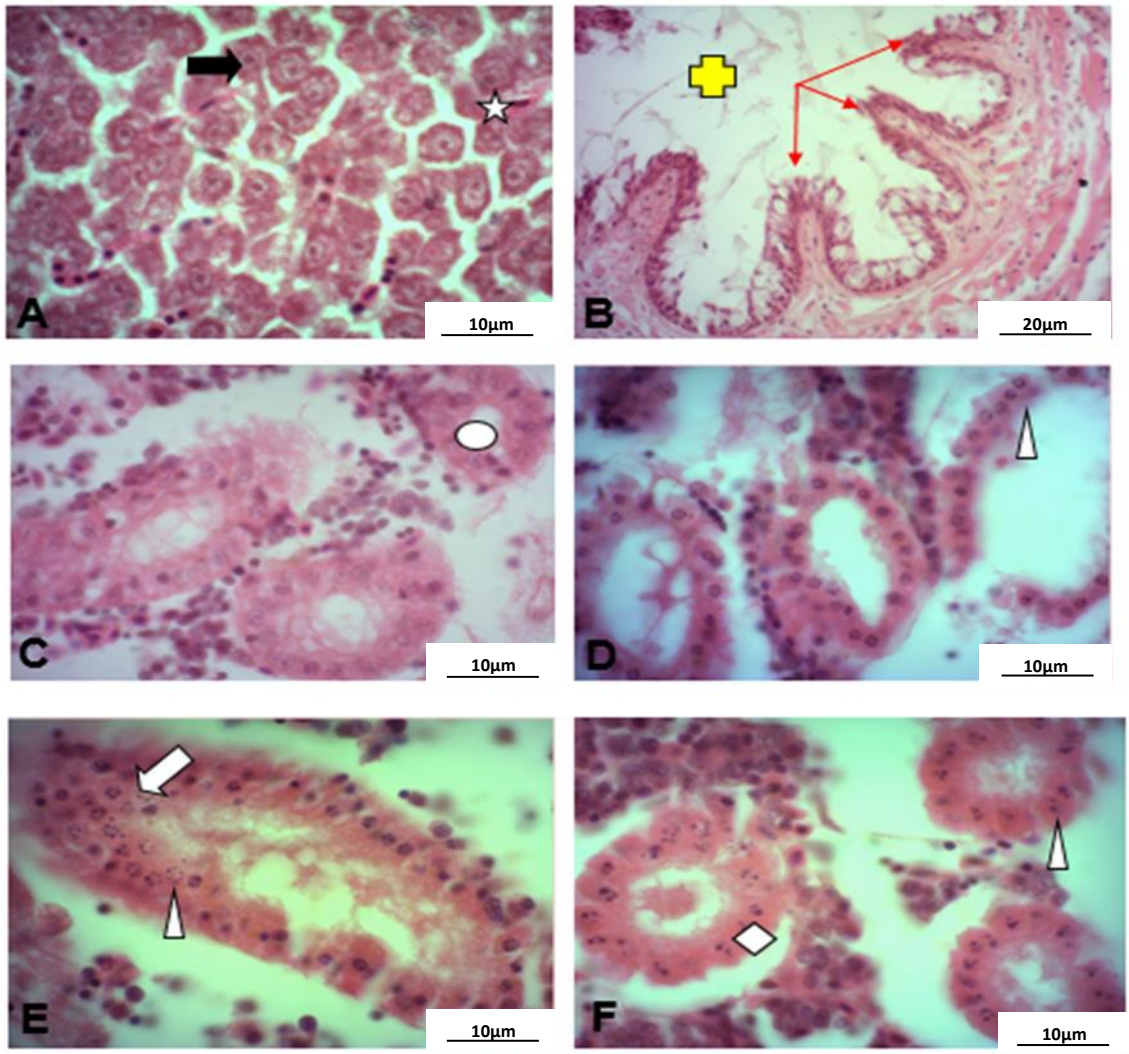


Figure 3-9: A) 1000x Liver section of adult zebrafish showing normal glycogen-rich liver scale 10 µm (black arrow), the sinusoids (white star) are usually one red blood cell thick. B) 400x Intestine section of adult zebrafish stained with H&E showing normal tissue with the scale of 10 µm. Trunk kidneys sections of adult zebrafish stained with H&E on scale 10 µm showing lumen (Yellow cross and gut curves (red arrows). C) 1000x normal trunk kidney tissue (white circle), D) 1000x trunk kidney tissue with some indications of necrosis karyorrhexis in fish exposed to 5 & 10 µg/g MC-LR. E&F) 1000x trunk kidney tissue with some indications of necrosis karyorrhexis (white triangle), hypertrophy (white Diamond) and hyperplasia (white arrow) in fish exposed to 5 & 10 µg/g *M. aeruginosa.*

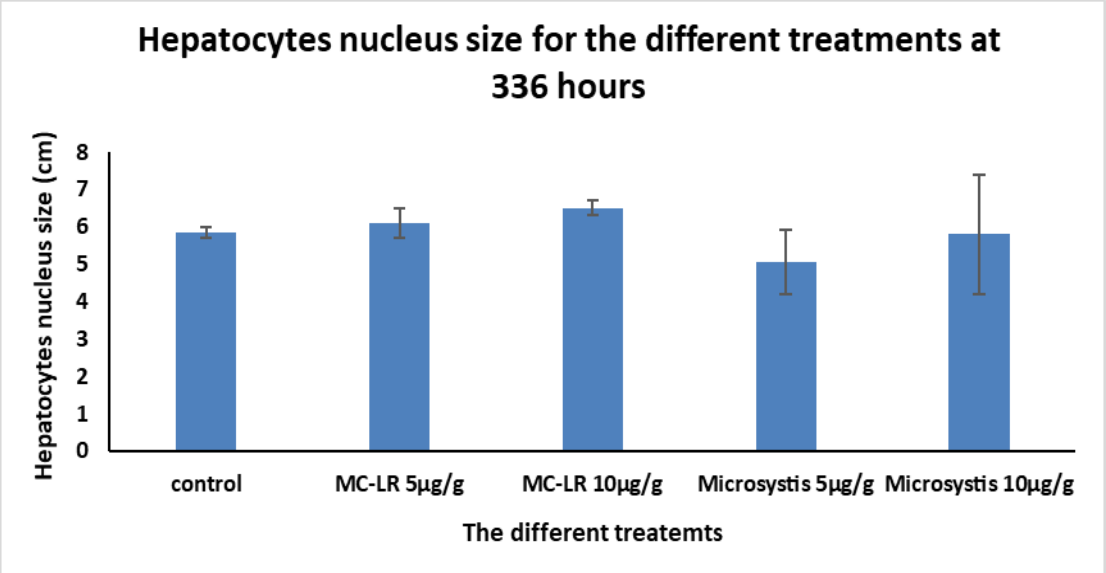
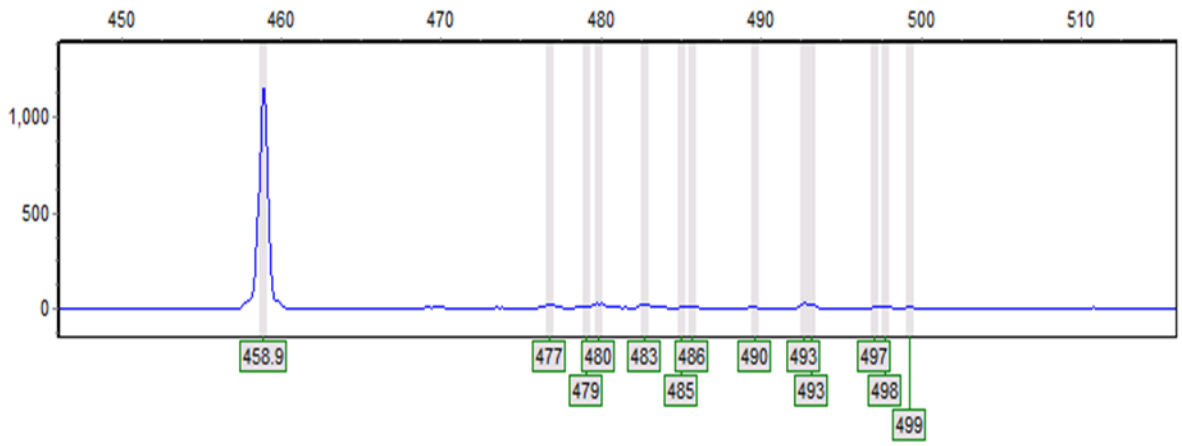


Figure 3-9 G: Hepatocytes nucleus size of the liver samples after dietary exposure adult zebrafish to different concentrations of MC-LR and *M. aeruginosa* at 336 hours, 3 replicates for each treatment / two fish for each replicate. The results were represented by (mean ± S.E).

3.3.4. Gut microbiota

Dietary exposure of *M. aeruginosa* / MC-LR revealed significant effects and shifting in the gut microbiota community. The analysis indicated that the different dietary treatments led to changes in microbial communities that grouped to some extent according to treatments (Figure 3-10 C). The results clearly showed that *Microcystis* 10µg/g was significantly different from the control (R= 0.47, P= 0.006), but *Microcystis* 5µg was not different from the control (R= 0.37, P= 0.004). In addition, the results confirmed significant differences between *Microcystis* 5µg/g and 10µg/g (R= 0.43, P= 0.022). *Microcystis* 10µg/g was significantly different from MC-LR 5µg/g (R= 0.39, P= 0.016) and MC-LR 10µg/g (R= 0.54, P= 0.006) (Figure 3-9 B). The electropherograms results (Figures 3-10 A 1&2) showed the control and one of the treatments of *Microcystis* 10µg/g. The individual peaks were represented the t-RFLP data that was received from Genewiz. The results for the electropherograms confirmed that there was a different response by showing different peaks in (Figure 3-10 A 2) in comparison to the control group (Figure 3-10 A 1).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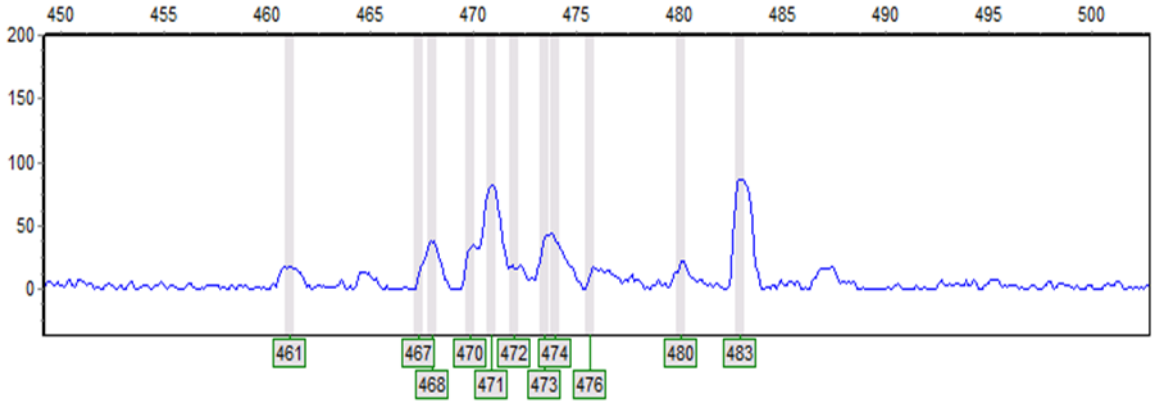
A (1)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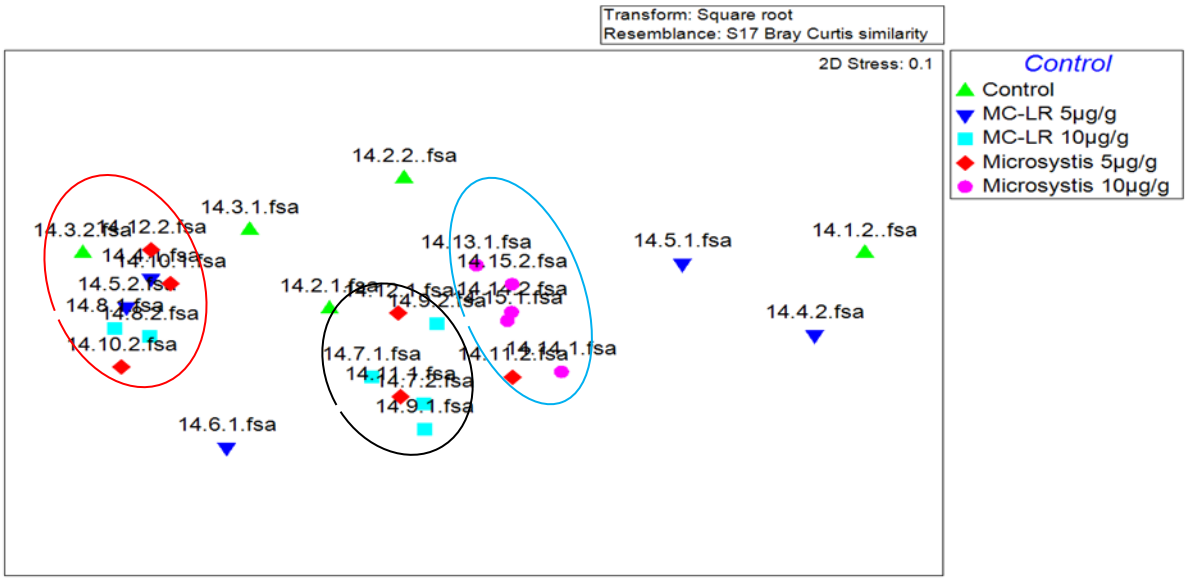
A (2)


Transform: Square root
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.1
Control
Control
MC-LR 5µg/g
MC-LR 10µg/g
Microsystis 5µg/g
Microsystis 10µg/g
14.2.2..fsa
14.3.1.fsa
14.3.2.fsa
14.12.2.fsa
14.10.1.fsa
14.5.2.fsa
14.8.1.fsa
14.8.2.fsa
14.10.2.fsa
14.2.1.fsa
14.12.1.fsa
14.9.2.fsa
14.15.1.fsa
14.13.1.fsa
14.15.2.fsa
14.14.2.fsa
14.7.1.fsa
14.11.1.fsa
14.7.2.fsa
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14.5.1.fsa
14.1.2..fsa
14.4.2.fsa

B

Global Test

Sample statistic (Global R): 0.191
Significance level of sample statistic: 1.7%
Number of permutations: 999 (Random sample from a large number)
Number of permuted statistics greater than or equal to Global R: 16

Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
Control, MC-LR 5ug/g	-0.021	43.5	462	462	201
Control, MC-LR 10ug/g	0.078	18.2	462	462	84
Control, Microsystis 5µg/g	0.004	37.4	462	462	173
Control, Microsystis 10µg/g	0.469	0.6	462	462	3
MC-LR 5ug/g, MC-LR 10ug/g	0.099	21.4	462	462	99
MC-LR 5ug/g, Microsystis 5µg/g	0.059	27.7	462	462	128
MC-LR 5ug/g, Microsystis 10µg/g	0.392	1.6	126	126	2
MC-LR 10ug/g, Microsystis 5µg/g	-0.02	35.9	462	462	166
MC-LR 10ug/g, Microsystis 10µg/g	0.539	0.6	462	462	3
Microsystis 5µg/g, Microsystis 10µg/g	0.432	2.2	462	462	10

C

Figure 3-10: A) An electropherogram from a control sample A (1) and one of the treatments A (2) (Genemaker software, softGenetics, PA), individual peaks represent t-RFLP. The grey bars represent the bins and squares below each peak represent allele labels that contain information on the allele calls, size and height of each peak. B) ANOSIM results (Primer6 software) presenting the differences in bacterial community structure among different treatments (MC-LR 5 µg/g blue upside-down triangle), (MC-LR 10 µg/g light blue square), (*Microcystis* 5 µg/g red Dimond), and (*Microcystis* 10 µg/g pink circle), through using t-RFLP analysis which showing the variability and similarity which representing by 3 main groups of community. Blue oval represents 10 µg/g *M. aeruginosa* that grouped separately, black oval represents 10 µg/g MC-KR that grouped separately, and red oval represent 5 µg/g *M. aeruginosa* and 5 µg/g MC-LR that grouped separately. C) ANOSIM results showed that R=0.2 which mean there is an indication of a separation among the levels of treatments, however the separation is not very high, as the R value is close to 0 with (P value=0.01). The pairwise test showed the differences among the different treatments, and the permutations (possible and actual) more than 400 which mean ANOSIM test is suitable to run the samples from the different treatments, as the acceptable value to run ANOSIM should be more than 100. In addition, the pairwise test indicate significate differences among the different treatments.

3.4. Discussion

3.4.1. Gross observations

Previous studies found no effects on fish growth or survival after 57 days in threadfin shad after exposing to *M. aeruginosa* at 4.4 and 10.0 µg MC-LR /g (Acuna et al., 2012) or after 60 days in medaka after exposing to MC-LR at 0.46, 0.85, 2.01 and 3.93 µg MC-LR/g dry diet (Deng et al., 2010). The results of the present study confirmed the previous results, as the gross observation was expected based on the too short exposure duration. Also, because of the short-term exposure, which was not enough to shift the energy that support the fish growth.

3.4.2. Gene expression

- **Oxidative stress-related genes expression:**

CAT gene expression showed no significant effects during the exposure time except significant response at 24 and 48 hours. However, *GPx* and *SOD1* revealed significant effects among different treatments in relation to the time (336 hours) with significant effects with some treatments at early time exposure. Up to date, no previous record exists regarding oxidative stress-related gene and the sub-lethal dietary exposing adult zebrafish to either MC-LR or *M. aeruginosa* to make a comparison with it. However, previous studies showed that the aqueous exposure to MC-LR or *M. aeruginosa* could affect the enzyme activity of the oxidative stress on zebrafish. Liu et al. (2014), showed that after aqueously exposing adult zebrafish to three different doses of MC-LR (1, 5 and 20 µg/L) for 30 days, the activities of antioxidant enzymes SOD, CAT and GPx significantly declined in the groups that treated with 5 and 20 µg/L MC-LR, which also confirming that the oxidative stress has an important role in the toxic mechanism of MC-LR.

The results of the present study confirmed that the effects of MC-LR or *M. aeruginosa* could be on the level of gene expression, as there was significant induction with especially *GPx* and *SOD1* within different times of the exposure. However, a study by Hou et al. (2014) showed that when female zebrafish were acutely exposed to MC-LR through (i.p.) injected at concentrations of 50 and 200 µg/kg body weight MC-LR, the levels of antioxidant enzymes CAT, SOD and GPX were increased, which possibly indicated that the occurrence of oxidative stress. The comparison among these two previous studies and the results of the present study showed that the higher acute doses through the i.p.

injection (Hou et al., 2014), induced the oxidative stress-related enzyme activities, while the opposite happened with the chronic aqueous exposure (Liu et al., 2014) and the dietary exposure in the present study showed an induction in gene expression. Hence, oxidative stress could be induced very early or later on depending on the dose, the way of exposure and the duration of the exposure. Besides that, the present study showed that the oxidative stress might be another possible toxic pathway for the sub-lethal or long-term exposure to MC-LR, as the protein phosphatase pathway could occur with the acute exposure to MC-LR.

- **Biotransformation related genes expression:**

The changing in the biotransformation related genes expression had been investigated in the context of enzyme activity and gene expression. *CYP1A1* was induced significantly in the early time's points of exposure to the different treatments and later at 336 hours, especially with MC-LR treatments, but down-regulated at 96 hours. Cytochrome P450 (*CYP1A1*) and glutathione-S-transferase1 (*GST1*) gene expression were not evaluated in the context of the toxicity of the sub-lethal dietary exposure to either *M. aeruginosa* or MC-LR. Similar to the results of the present study, Zaho et al. (2015) were exposing female zebrafishfish to MC-LR in concentrations 2, 10 and 50 µg/L for 21 days. The results showed that *CYP19A*, *CYP19B* and *CYP17* gene expression were induced after the exposure to MC-LR at dose 10 µg/L. The results of the present study suggested that MC-LR altered *CYP1A1* on the level of gene expression and *CYP1A1* works to detoxicate MC-LR, as *CYP1A1* represent detoxification phase 1 in the liver.

Furthermore, *GST1* revealed significate induction among different treatments in relation to the time 336 hours with significant effects with some treatments at early time exposure. Hou et al. (2014) exposed zebrafish females acutely to MC-LR through (i.p.) injected to doses of 50 and 200 µg/kg body weight MC-LR. The results showed that the levels of antioxidant enzymes CAT, SOD, GPx and especially the biotransformation enzyme GST were increased. In comparing to the results of the present study, *GST1* represent detoxification phase 2 in the liver and the induction in the present study confirms that MC-LR alters *GST1* on the level of gene expression and in the context of enzyme activity. (Hou et al., 2014). *GST1* induce together with *CYP1A1*, as *GST1* consider liver detoxification phase 2, which take the metabolisms form phase 1 and changing them, so they excreted easily from the body (Zhang et al., 2015).

- **Protein Phosphatase gene expression:**

The expression of *PPP1ca* appeared to be affected significantly by the different treatments. In addition, there was significant down-regulation with some of the MC-LR doses during the exposure time and overall, there was significant induction with some treatments of *M. aeruginosa.* Although *PPP1ca* is related to MC-LR toxicity, the expression of this gene has not been measured previously. Protein phosphatase 1 (PP1) and PP2A together belong to the PPP family, which is contributing most of the serine/threonine phosphatase activity in cells. So far, MC-LR is considered an active inhibitor of PP2A/PP1, which may lead to proteins hyperphosphorylation and this can be explained by the MC-LR toxicity and tumour promotion activity (Wang et al., 2010). Also, MC-LR can inhibit PP2A more than PP1, as MC-LR is high affinity (Xing et al., 2006). Consequently, decreasing PP2A activity by MC-LR exposure could alter series cellular effects such as cell cycle, cell proliferation, division, signal transduction and gene expression (Sun et al., 2014). Changes in protein phosphatase PP2 have been investigated in the context of gene expression in zebrafish after exposing to *M. aeruginosa* / MC-LR.

A study by Wang et al. (2010), showed that after aqueously exposing adult zebrafish to MC-LR at concentrations 2 or 20 mg/L for 30 days, *PP2aA* and *PP2aC* genes were very slightly increased to nearly 1.5 fold change. On the other hand, Tzima et al. (2017), used zebrafish larvae and exposed these to 50 and 500 µg/L MC-LR for four days. The results showed that 40% reduction of PP2A enzyme activity in comparing to the controls, which may be indicated to the early effects of MC-LR. These results indicate that PP2 could induce early and late in response to MC-LR toxicity. In addition, preliminary information from proteomics studies indicated that chronic toxicity of MC-LR was different from acute toxicity, and oxidative stress could be the main toxic pathway instead of disruption of protein phosphatases (Chen et al. 2016).

The results of gene expression profiles of the present study suggested that the timing of changes in gene expression differs among the different treatments and it is important to consider in the context of the toxicity of MC-LR and *M. aeruginosa*. So far, this is the only study that has investigated changes in expression of *PPP1ca* gene expression in adult zebrafish overtime during a dietary exposure to MC-LR or *M. aeruginosa*. Also, the results of the present study showed that the oxidative stress might be another possible toxic pathway for the sub-lethal or long-term exposure to MC-LR, as the protein

phosphatase pathway could occur with the acute exposure to MC-LR. Another possibility that the results of the present study regarding *PPP1ca* gene expression showing that the toxicity of MC-LR could happen via protein phosphatase route and MC-LR effects could inhibit *PPP1ca* on the level of gene expression.

3.4.3. Histopathology

The liver is considered the main target for MC-LR (Carmichael, 1995). Besides that, MC-LR could target other organs such as kidney, gills and the gastrointestinal tract (Rabergh et al., 1991; Kotak et al., 1996; Carbis et al., 1997) and the reproductive system in mice (Ding et al., 2006), rat (Li et al., 2008; Xiong et al., 2009) and fish (Baganz et al., 1998). The histopathology results of the liver samples showed no significant effects on the hepatic nucleus size and minor lesions observed in the liver tissue were consistent with normal variation of control fish livers.

No previous report exists regarding sublethal dietary exposing adult zebrafish to either MC-LR or *M. aeruginosa* and their effects on the liver histopathology and to make a comparison with the results of the present study. However, a previous study with threadfin shad and longer (chronic) dietary exposure of *M. aeruginosa* at 4.4 and 10.0 µg MC-LR /g for 57 days reported lesions in the liver such as severe glycogen depletion, eosinophilic droplets, single-cell necrosis and sinusoidal congestion after exposure to *Microcystis* (Acuna et al., 2012). The different in results between the present study and Acuna et al. (2012) results could be due to the short-term of exposure that was adopted in the present study. Moreover, there were minor lesions detected, which means the effects of either MC-LR or *M. aeruginosa* started to build up by the time.

MC-LR requires uptake through the bile acid transport system, as MC-LR is not cell permeant. The bile acid transport system is presented by the hepatocytes and cells lining in the small intestine (Dawson, 1998). Ito et al. (2000) confirmed that orally taken MC-LR was mainly absorbed in the small intestine. Additionally, by the portal vein, the small intestine absorbs MC-LR as well as the metabolic products of gut microflora and transport these from gut to liver and other organs by blood (Goel., 2014). The results of the present study showed that the gut samples revealed no histopathological changes with either MC-LR or *M. aeruginosa* treatments (Figure 3-2B). Similarly, Acuna et al. (2012) used threadfin shad and exposed them to *Microcystis* at concentrations of 4.4 and 10.0 µg MC-LR /g for a long dietary exposure of 57 days. Their results showed no lesions were

detected in the intestine after the dietary exposure. The results of the present study suggested that the main effects of either MC-LR or *M. aeruginosa* were in the gut microbiota community, as the results showed in section 3.3.4. and not on the level of the histopathological changes of the gut tissue.

In the present study, the trunk kidney showed indications of necrosis (karyorrhexis) in fish exposed to 5 & 10 µg/g MC-LR, plus, karyorrhexis, hypertrophy and hyperplasia were observed in fish exposed to 5 & 10 µg/g *M. aeruginosa*. No previous record to make a comparison with and probably these effects can be related to early effects of MC-LR and *M. aeruginosa* and it seems that the trunk kidney was more sensitive than the liver and the gut. Additionally, the gene expression results for the present study showed that the biotransformation genes expression of the liver detoxification phase 1 *CYP1A1* and liver detoxification phase 2 *GST1* significant response during the time course with the different treatments. This is Another possibility that why the kidney showed histopathological changes to either MC-LR and *M. aeruginosa,* because of the liver phase 1 by *CYP1A1* metabolism MC-LR and *M. aeruginosa* and then the liver phase 2 by *GST1* take the toxic substances from phase 1 and conjugated with glutathione via GST specific enzyme and then excreted from the body by bile or urine (Zhang et al., 2015).

3.4.4. Gut microbiota

According to Roeselers et al. (2011) all stages of the zebrafish life cycle showed that the gut microbiota are numerically dominated at by members of the bacterial phylum Proteobacteria, with the phyla Firmicutes and Fusobacteria also prevalent during larval and adult stages, respectively. Few previous studies exist that address the effects of either MC-LR or *M. aeruginosa* on the gut microbiota in few organisms such as rats, and mice (Lin et al., 2015 & Chen et al., 2015). According to Roeselers et al. (2011), the gut microbiota of laboratory-reared zebrafish is similar in composition to zebrafish collected recently from their natural habitat. A study by Lin et al. (2015) showed that the short-term dietary exposure to MC-LR had made a significant decline in the composition of functional genes in rat gut. As they designed their study to reveal the shift in the microbial functional genes in the rut gut. Similarly, a recent study by Chen et al. (2016) examined the effects of sub-chronic MC-LR on gut microflora in different gut regions of mice. After intragastric administration of MC-LR, Chen et al. (2016) used Denaturing Gradient Gel Electrophoresis (DGGE) method to record and to profile the shifting of the microbiota. Their results showed an increase in the microbial species richness in caecum

and colon. Besides that, MC-LR disturbs the balance of the gut microbiota and the toxicological effects varied between the jejunoileum and the other two gut regions of the mice gut. Also, the results of the present study showed that MC-LR and *M. aeruginosa* can affect the zebrafish gut physiology and can disrupt the microbial community in the intestine and this can consider a vital toxicology indicator to either MC-LR or *M. aeruginosa*.

So far, no study to our knowledge has liked at the effects of sub-lethal dietary effects of MC-LR and *M. aeruginosa* on zebrafish's gut microbiota community, because the response can be considered a vital sight to investigate the toxicity of MC-LR and *M. aeruginosa.* Overall, the results of the present study showed that *Microcystis* 10 µg/g had a substantial effect on gut microbiota community in comparison to the other treatments and the control. The gut microbiota results showed that there were some treatment-related changes in the gut microbial communities for MC-LR and *M. aeruginosa* with significant effects through grouping the community as it showed in (Figures 3-10 A-C).

Chapter 4

General discussion and conclusions

Toxin-producing *M. aeruginosa* blooms are widespread in many aquatic ecosystems throughout the world. In this project, the overall aim was to investigate the effects of aqueous and dietary exposures of *M. aeruginosa* / MC-LR at environmentally relevant concentrations on zebrafish. First, gene expression profiles (oxidative stress group, biotransformation group, protein phosphatase and vitellogenin gene) were investigated according to aqueous treatment concentration and duration of exposure; and second histopathology (liver and gut) was examined. Overall, no previous study has investigated gene expression profile at dose and time relationships after exposure of zebrafish larvae to either MC-LR or *M. aeruginosa* and no previous study has performed any histopathological studies on zebrafish larvae after aqueous exposure to either MC-LR or *M. aeruginosa*. The third and the fourth targets of this project were to investigate the effects of sub-lethal dietary exposure of *M. aeruginosa* / MC-LR on adult zebrafish on gene expression profiles for (oxidative stress group, biotransformation group and protein phosphatase), histopathology for whole adult fish (male and female) and the gut microbiota community. Up until now, the present study is the first study that has investigated gene expression and gut microbiota community on adult zebrafish after dietary exposure to either MC-LR or *M. aeruginosa*.

The results of the present study provide a new insight that MC-LR / *M. aeruginosa* affected the gut physiology and disrupted the microbial community in the intestine. Furthermore, the gut microbiota community were grouped regarding the different treatment concentrations. This can be considered an important toxicology indicator to either MC-LR or *M. aeruginosa*. No previous report exists in order to compare the results of the present study with it. For further investigations, gut microbiota sequencing will be the next step, to get a better understanding of the changes in the microbiota community and the consequences on general health. Also, longer dietary exposure is required to see the differences in the gut microbiota community between the sub-lethal and the chronic dietary exposure to either MC-LR or *M. aeruginosa*. Additionally, a further investigation of the effect of dietary exposure of other MC congeners on zebrafish gut microbiota community is needed.

To date, the present study is the only one study that has performed the histological in general and plastic histology in particular in zebrafish larvae after exposure to either MC-LR or *M. aeruginosa*. In the present study, the main focus regarding the histological features was on the whole larvae, particularly the liver and the gut, since the liver is

considered the main target for MC-LR and the gut is crucial to see the histological changes after exposure to either MC-LR or *M. aeruginosa*. MC-LR is not cell permeant, as it required uptake by the bile acid transport system, which is present in the cells lining in the small intestine and in the hepatocytes and (Dawson, 1998). Additionally, the results of the present study showed that no appreciable histological changes in either the hepatocytes or in the gut regarding MC-LR different doses. However, the higher dose of *M. aeruginosa* 400 µg/L showed that the liver tissue had evacuation and tissue damage, which might be due to the effects of the other substances in *M. aeruginosa*. Further questions remain to understand the absorbent mechanism by the gut and to track the effects of *M. aeruginosa* on the hepatocytes by using transgenic zebrafish. Also, to test different congeners of MC to examine the effects on the gut and the liver.

The liver is considered the main target for MC-LR (Carmichael, 1995). Besides that, MC-LR might target other organs such as kidney, gills and the gastrointestinal tract (Rabergh et al., 1991; Kotak et al., 1996; Carbis et al., 1997) and the reproductive system in fish (Baganz et al., 1998). The histopathological results of the present study showed that the sub-lethal dietary exposure of MC-LR or *M. aeruginosa* showed effects on the trunk kidney, however the liver samples showed no significant effects on the hepatocytes nucleus size and minor lesions observed in the liver tissue were consistent with normal variation of control fish livers. No previous report exists regarding sublethal dietary exposing adult zebrafish to either MC-LR or *M. aeruginosa* and their effects on the liver histopathology and to make a comparison with the results of the present study. A previous report by Acuna et al. (2012) with threadfin shad and longer (chronic) dietary exposure of *M. aeruginosa* reported no lesions in gut or kidney, however they reported significant effect on the liver tissue such as severe glycogen depletion, eosinophilic droplets, single-cell necrosis and sinusoidal congestion. The disagreement of the present study with Acuna et al. (2012) results, is possibly due to the short-term of exposure that was adopted in the present study. Also, there were minor lesions detected in the liver samples, which may mean that the effects of either MC-LR or *M. aeruginosa* started to build up by that time. Future work will be required to understand in more depth why this early effect was occurred in the trunk kidney. Also, more histological work will be required to investigate effects on testis and the ovaries. Using a fluorescence microscope and the Transmission Electron Microscope (TEM) would be advantageous to track the effects of MC-LR / *M. aeruginosa* during and after the dietary exposure for the zebrafish to gain a better understanding on histopathological changes on the gut, liver, kidney and gonads.

The investigation of the present study showed that protein phosphatase gene expression was significantly affected and this contribute to the understanding of how protein phosphatases are affected by MC-LR exposure in fish. Protein phosphatase 1 and 2 are critical regulators of the MC-induced molecular network (Liu and Sun, 2015). The results of the present study confirm previous knowledge and add new information regarding larvae and adult zebrafish after aqueous or dietary exposure respectively. The results showed that the *PPP1ca* gene expression turns on in the early time points with low doses of MC-LR, which probably mean more mRNA of *PPP1ca* was produced and it is unknown if its activity is changed or not. In addition, this induction is consistent with enhancing enzyme activity at this time point, as well as with previous studies. The implications are perhaps this induction of *PPP1ca* enables the cells to keep maintaining enzyme activity of protein phosphorylation under these conditions. Also, the down-regulation of *PPP1ca* gene expression that happened with higher doses confirm the occurrence of hyperphosphorylation and increasing ROS, which was confirmed by the results of oxidative stress-related genes about time and dose response. To date, the present study is the only one that exists regarding the effects of MC-LR on *PPP1ca* gene expression during the time course. Besides that, no previous studies have used time relation effects of MC-LR and *M. aeruginosa* on adult and larval zebrafish to make the comparison. A further investigation needs to understand in more depth how this mechanism happens.

This present study demonstrated that *VTG1* gene expression was induced and this presents a different prospective on the oestrogenicity of *M. aeruginosa* exposure. The results of the present study showed that MC-LR and *M. aeruginosa* have estrogenic effects on zebrafish larvae on time or concentration response, particularly the early time points and later of the exposure. Oziol and Bouaïcha (2010) had similar results to the findings of the present study, as their result showed that a low level of estrogenic response in the human breast carcinoma cell line when cells were exposed to purified MC-LR. In contrast, the results of the present study both agreed and disagreed with the results of Rogers et al. (2011). Their results showed that MC-LR did not induce *VTG1* however *M. aeruginosa* induced *VTG1* in larval zebrafish. Conversely, the results of the present study showed that *VTG1* was induced in larval zebrafish exposed to both MC-LR and *M. aeruginosa*, which indicated that the estrogenic receptor mediated induction of *VTG1* in zebrafish was activated by MC-LR and *M. aeruginosa*. Future investigation is needed to understand the mechanism of the potential for endocrine disruption that could be added

to the environmental effects as well as the consequence on the public health concerns related to bloom events.

MCs could alter the antioxidant system and induce oxidative stress in diverse aquatic species and different organs. Generally, MC-LR is considered to inhibit protein phosphatases besides its effects on oxidative stress. MC exposure may lead to an extreme formation of reactive oxygen species (ROS), which may lead to oxidative damage. The results of the present study for the aqueous and the sub lethal dietary exposure provide an insight to the changes that happened to the gene expression level in adult and larval zebrafish. The recent reports were focusing on enzyme activity and the aqueous exposure. Previous reports suggested that there is a connection between cellular hyperphosphorylation state and oxidative stress generation induced by MCs exposure. Furthermore, a hyperphosphorylated cellular environment induced by MCs exposure might alter antioxidant enzymes, contributing to the generation of oxidative damage (Amado and Monserrat, 2009b). The present study is the first study that investigated the effects of dietary and aqueous exposures of MC-LR and *M. aeruginosa* on adult and larval zebrafish respectively of the level of gene expression of oxidative stress. The results of the present study confirmed that MC-LR / *M. aeruginosa* have significant effects on some of the genes related to the oxidative stress and altered them with the different treatments of MC-LR and *M. aeruginosa* in the early and late time points. Hence, the present study showed that the oxidative stress might be another possible toxic pathway for sub-lethal or long-term exposure to MC-LR, as the protein phosphatase pathway could occur with the acute exposure to MC-LR. More work is required to investigate a longer term dietary exposure of MC-LR and *M. aeruginosa* on adult zebrafish to understand these pathways in more depth.

Up until now, the present study is the first study that investigated the effects of MC-LR or *M. aeruginosa* on biotransformation related genes expression on zebrafish larvae. Also, cytochrome P450 (CYP1A1) and glutathione-S-transferase1 (GST1) gene expression were not evaluated in the context of the toxicity of the sub-lethal dietary exposure to either *M. aeruginosa* or MC-LR. The results of the present study regarding the sub-lethal dietary exposure suggested that MC-LR altered *CYP1A1* and *GST1* on the level of gene expression and this induction of CYP1A1 and GST1 to work to detoxification MC-LR, as CYP1A1 and GST1 represent detoxification phase 1 and 2 in the liver respectively. Moreover, in early time points of the aqueous exposure zebrafish

larvae to either *M. aeruginosa* or MC-LR showed that MC-LR and *M. aeruginosa* significantly altered *CYP1A1* and *GST1* and this induction of *CYP1A1* and *GST1* showing the early effects of MC-LR and *M. aeruginosa* and how fast the response was from the biotransformation system to detoxification MC-LR, as CYP1A1 and GST1 represent detoxification phase 1 and 2 in the liver respectively. Future plans would be required to investigate in depth the effects of the long term of dietary exposure of MC-LR and *M. aeruginosa* on adult zebrafish.

Finally, other future work is required to reveal the mechanism of the parental transmission of MC-LR / *M. aeruginosa* toxicity in normal and transgenic adult zebrafish after the dietary exposure. Also, further investigation will need to perform by fluorescence microscope on transgenic zebrafish embryos and larvae to track the effects of either MC-LR or *M. aeruginosa*. Then check the effects on the surviving larvae, after moving them to clean water.

References

- Abdel-Rahman, S.; El-Ayouty, Y. and Kamael, H. 1993. Characterization of heptapeptide toxins extracted from *Microcystis aeruginosa* (Egyptian isolate). *International Journal of Peptide and Protein Research*, 41, 1–7.
- Acuna, S.; Baxa, D. and Teh, S. 2012. Sub-lethal dietary effects of microcystin producing *Microcystis* on threadfin shad, *Dorosoma petenense*. *Toxicon.*, 60(6), 1191-202.
- Adámek, Z.; Sikora, J.; Bláha, L. and Marˇsálek, B. 2011. Screening assessment of cyanobacterial embryotoxicity to Japanese Medaka, *Oryzias Latipes* (Actinopterygii: Beloniformes: Adrianichthyidae). *Acta. Ichthyol. Piscat*., 41, 293–299.
- Adamovsky, O.; Kopp, R.; Hilscherova, K.; Babica, P.; Palikova, M. and Paskova, V. 2007. Microcystin kinetics (bioaccumulation and elimination) and biochemical responses in common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmic hthysmolitrix*) exposed to toxic cyanobacterial blooms. *Environ. Toxicol. Chem*., 26(12), 2687-2693.
- Aguirre-Martínez, G.; Reinardy, H.; Martín-Díaz, M.; Henry, T. 2017. Response of gene expression in zebrafish exposed to pharmaceutical mixtures: Implications for environmental risk. *Ecotoxicology and Environmental Safety*, 142, 471–479.
- Amado, L. and Monserrat, J. 2009b. Oxidative stress generation by microcystins in aquatic animals: Why and how. *Environment International*, 36:226–235.
- Amado, L.; Garcia, M.; Ramos, P.; Freitas, R.; Zafalon, B. and Ferreira, J. 2009a. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity. *Sci. Total Environ.*, 407, 2115–23.

- Ankley, G.; Jensen, K.; Kahl, M.; Makynen, E.; Blake, L.; Greene, K.; Johnson, R. and Villeneuve, D. 2009. Ketoconazole in the fathead minnow (*Pimephales promelas*): reproductive toxicity and biological compensation. *Environmental Toxicology and Chemistry*, 26, 1214–1223.

- Aravindakshan, J.; Paquet, V.; Gregory, M.; Dufresne, J.; Fournier, M.: Marcogliese,D. and Cyr, D. 2004. Consequences of xenoestrogen exposure on male reproductive function in spot tail shiners (*Notropis hudsonius*). *Toxicological Sciences,* 78:156–165.

- Azevedo, S.; Carmichael, W.; Jochimsen, E.; Rinehart, K.; Lau, S. and Shaw, G. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru–Brazil. *Toxicology*, 181–182, 441–6.

- Baganz, D.; Staaks, G. and Steinberg, C. 1998. Impact of the cyanobacteria toxin, microcystin-LR on behaviour of zebrafish, *Danio rerio. Water Res*., 32, 948–952.

- Best, J.; Eddy, F. and Codd, G. 2003. Effects of *Microcystis* cells, cell extracts and lipopolysaccharide on drinking and liver function in rainbow trout *Oncorhynchus mykiss walbaum*. *Aquat. Toxicol*., 64, 419–426.

- Best, J.; Eddy, F.; Codd, G. 2001. Effects of purified microcystin-LR and cell extracts of *Microcystis* strains PCC 7813 and CYA 43 on cardiac function in brown trout (*Salmo trutta*) alevins. *Fish Physiol. Biochem*., 24 (3), 171–178.

- Best, J.; Pflugmacher, S.; Wiegand, C.; Eddy, F.; Metcalf, J. and Codd, G. 2002. Effects of enteric bacterial and cyanobacterial lipopolysaccharides, and of MC-LR, on glutathione S-transferase activities in zebra fish (*Danio rerio*). *Aquat. Toxicol*., 60, 223–231.

- Black, J.; Maccubbin, A. and Shiffert, M. 1985. A reliable, efficient microinjection apparatus and methodology for the in vivo exposure of rainbow trout

and salmon embryos to chemical carcinogens. *J. Natl. Cancer Inst*., 75, 1123–1128.

- Boopathi, T. and Ki, J. 2014. Impact of environmental factors on the regulation of cyanotoxin production. *Toxins (Basel)*, 6(7), 1951-78.

- Brooks, W. and Codd, G. 1987. Immunological and toxicological studies on *Microcystis aeruginosa* peptide toxin. *Br. Phycol. J*., 22, 301.

- Brugman, S. 2016. The zebrafish as a model to study intestinal inflammation. *Developmental and Comparative Immunology*, 64, 82-92.

- Bu, Y.; Li, X.; Zhang, B.; Chung, I. and Lee, J. 2006. Microcystins cause embryonic toxicity in mice. *Toxicon*., 48, 966–972.

- Buryskova, B.; Hilscherov´a, K.; Babica, P.; Vrˇskov´, D.; Marˇs´alek, B.; Bl´aha, L. 2006. Toxicity of complex cyanobacterial samples and their fractions in *Xenopus laevis* embryos and the role of microcystins. *Aquatic Toxicology*, 80, 346–354.

- Byrd, C. and Brunjes, P. 1995. Organization of the olfactory system in the adult zebrafish: Histological, immunohistochemical, and quantitative analysis. *J. Compar. Neurol*., 358, 247–259.

- Caminos, E.; Velasco, A.; Jarrin, M.; Lillo, C.; Jimeno, D.; Aijon, J. and Lara, J. 2000. A comparative study of protein kinase C-like immunoreactive cells in the retina. *Brain Behav. Evol*., 56, 330–339.

- Campos, A. and Vasconcelos, V. 2010. Molecular mechanisms of microcystin toxicity in animal cells. *Int. J. Mol. Sci*., 11, 268–287.

- Carbis, C.; Mitchell, G.; Anderson, J. and McCauley, I. 1996a. The effects of microcystins on the serum biochemistry of carp, *Cyprinus carpio L*., when the toxins are administered by gavage, immersion and intraperitoneal routes. *J. Fish Dis.*, 19(2), 151-159.

- Carbis, C.; Rawlin, G.; Grant, P.; Mitchell, G.; Anderson, J. and McCauley, I. 1997. A study of feral carp, *Cyprinus carpio* L., exposed to *Microcystis aeruginosa* at Lake Mokoan, Australia, and possible implications for fish health. *J. Fish. Dis*., 20, 81–91.

- Carbis, C.; Rawlin, G.; Mitchell, G.; Anderson, J. and McCauley, I. 1996b. The histopathology of carp, *Cyprinus carpio L.*, exposed to microcystins by gavage, immersion and intraperitoneal administration. *J. Fish Dis*., 19 (3), 199-207.

- Carmichael, W. 1992. Cyanobacteria secondary metabolites- The cyanotoxins: A review. *J. Appl. Bacteriol*., 72, 445–459.

- Carmichael, W. 1995. *Toxic Microcystis and the environment*. In: Watanabe, M.; Harada, K.; Carmichael, W. and Fujiki, H. (Eds.), Toxic *Microcystis*. CRC Press, New York, pp. 1–11.

- Carmichael, W.; Azevedo, S.; An, J.; Molica, R.; Jochimsen, E.; Lau, S.; Rinehart, K.; Shaw, G.; Eaglesham, G. 2001. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. *Environ. Health Perspect.*, 109, 663–668.

- Cazenave, J.; Bistoni, M.; Pesce, S. and Wunderlin, D. 2006b. Differential detoxification and antioxidant response in diverse organs of Corydoras paleatus experimentally exposed to microcystin-RR. *Aquat. Toxicol*., 76, 1-12.

- Cazenave, J.; Bistoni, M.; Zwirnmann, E.; Wunderlin, D. and Wiegand, C. 2006a. Attenuating effects of natural organic matter on microcystin toxicity in zebra fish (*Danio rerio*) embryos benefits and costs of microcystin detoxication. *Environ. Toxicol.*, 21, 22–32.

- Cerda, J.; Conrad, M.; Markl, J.; Brand, M. and Herrmann H. 1998. Zebrafish vimentin: Molecular characterization, assembly properties and developmental expression. *Eur. J. Cell Biol.*, 77,175–187.

- Cheesman, S. and Guillemin, K. 2007. We know you are in there: conversing with the indigenous gut microbiota. *Res. Microbiol*., 158, 2-9.

- Chen, J., Xie, P., Li, L. and Xu, J. 2009. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. *Toxicol. Sci.*, 108(1), 81-9.

- Chen, J., Zhang, D., Xie, P., Wang, Q., Ma, Z. 2009. Simultaneous determination of microcystin contaminations in various vertebrates (fish, turtle, duck and water bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic Microcystis blooms. *Sci. Total. Environ*., 407, 3317–3322.

- Chen, J.; Xie, P.; Guo, L.; Zheng, L. and Ni, L. 2005. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in a freshwater snail (*Bellamya aeruginosa*) from a large shallow eutrophic lake of the subtropical China. *Environmental Pollution*, 134, 423–430.

- Chen, J.; Xie, P.; Lin, J.; He, J.; Zeng, C. and Chen, J. 2015. Effects of microcystin-LR on gut microflora in different gut regions of mice. *J Toxicol Sci.*, 40(4), 485-94.

- Chen, L., Chen, J., Zhang, X. and Xie, P. 2016. A review of reproductive toxicity of microcystins, *J. Hazard Mater*, 301, 381-99.

- Chen, L.; Zhang, X.; Zhou, W.; Qiao, Q. and Liang H. 2013. The interactive effects of cytoskeleton disruption and mitochondria dysfunction lead to reproductive toxicity induced by microcystin-LR. *PLOS ONE*, 8(1), 1-11.

- Chen, Y.; Lee, T.; Lee, S.; Huang, H.; Huang, R. and Chou, H. 2006. Comparison of protein phosphatase inhibition activities and mouse toxicities of microcystins. *Toxicon*, 47, 742–6.

- Chen, Y.; Xu, J.; Li, Y. and Han, X. 2011. Decline of sperm quality and testicular function in male mice during chronic low-dose exposure to microcystin-LR. *Reprod., Toxicol.*, 31, 551–557.

- Chomczynski, P.; Wilfinger, W. and Mackey, K. 2013. *Single-Step Method of Total RNA Isolation by Guanidine–Phenol Extraction in eLS*. John Wiley & Sons, Ltd.

- Codd, G.; Bell, S. and Brooks, W. 1989. Cyanobacterial toxins in water. *Water Sci. Technol.*, 2, 1-13.

- Codd, G.; Morrison, L. and Metcalf, J. 2005. Cyanobacterial toxins: risk management for health protection. *Toxicol. Appl. Pharmacol*., 203, 264–272.

- Connaughton, V.; Behar, T.; Liu, W. and Massey, S. 1999. Immunocytochemical localization of excitatory and inhibitory neurotransmitters in the zebrafish retina. *Vis. Neurosci.*, 16, 483–490.

- Cousins, I.: Bealing, D.; James, H. and Sutton, A., 1996. Biodegradation of microcystin-LR by indigenous mixed bacterial populations. *Water Res*., 30, 481–485.

- Cox, W. and Singer, V. 1999. A high-resolution, fluorescence-based method for localization of endogenous alkaline phosphatase activity. *J. Histochem. Cytochem.*, 47, 1443–1456.

- Davis, T.; Koch, F.; Marcoval, M.; Wilhelm, S. and Gobler, C. 2012. Mesozoo-plankton and microzooplankton grazing during cyanobacterial blooms in the western basin of Lake Erie. *Harmful Algae*, 15, 26–35.

- Dawson, R. 1998. The toxicology of microcystins. *Toxicon*., 36 (7), 953–962.

- De Figueiredo, D.; Azeiteiro, U.; Esteves, S.; Gonc¸alves, F. and Pereira, M. 2004. Microcystin-producing blooms – a serious global public health issue. *Ecotoxicology and Environment Safety*, 59: 151–163.

- De la Cruz, A.; Antoniou, M.; Pelaez, M.; Hiskia, A.; Song, W.; O'Shea, K.; He, X. and Dionysiou, D. 2011. Can we effectively degrade microcystins? Implications for impact on human health status. *Anti-Cancer Agents Med. Chem.*, 11(1), 19-37.

- Deblois, C.; Giani, A. and Bird, D. 2011. Experimental model of microcystin accumulation in the liver of *Oreochromis niloticus* exposed sub chronically to a toxic bloom of *Microcystis* sp. *Aquat. Toxicol*., 103, 63–70.

- DeMott, W. and Dhawale, S. 1995. Inhibition of in vitro protein phosphatase activity in three zooplankton species by microcystin-LR, a toxin from cyanobacteria. *Arch Hydrobiol*., 34, 417–24.

- Deng, D.; Zheng, K.; The, F.; Lehman, P. and The, S. 2010. Toxic threshold of dietary microcystin (-LR) for quart medaka. *Toxicon*., 55: 787–794.

- Detrich, H.; Westerfield, M. and Zon, L. 1999. Overview of the Zebrafish system. *Methods Cell Biol.*, 59, 3–10.

- Ding, W. and Ong, C. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol. Lett*., 2 (20), 1–7.

- Ding, W.; Shen, H. and Ong, C. 2000. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. *Hepatology*., 32, 547–55.

- Ding, W.; Shen, H. and Ong, C. 2001. Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. *Environ Health Perspect*., 108, 605–9.

- Ding, X.; Li, X.; Duan, H.; Chung, I. and Lee, J. 2006. Toxic effects of *Microcystis* cell extracts on the reproductive system of male mice. *Toxicon.,* 48, 973–979.

- Dittmann, E.; Fewer, D.P.; Neilan, B.A. 2013. Cyanobacterial toxins: Biosynthetic routes and evolutionary roots. *FEMS Microbiol. Rev*., 37, 23–43.

- Dong, G.; Zhu, X.; Han, D.; Yang, Y.; Song, L. and Xie, S. 2009. Effects of dietary cyanobacteria of two different sources on growth and recovery of hybrid tilapia (*Oreochromis niloticus x Oreochromis aureus*). *Toxicon*., 54(3), 208-216.

- Edwards, C.; Graham, D.; Fowler, N. and Lawton, L 2008. Biodegradation of microcystins and nodularin in freshwaters. Chemosphere, 7, 1315–1321.

- Elbekai, R. and El-kadi, A. 2005. The role of oxidative stress in the modulation of aryl hydrocarbon receptor-regulated genes by As3+, Cd3+, and Cr6+. *Free Radic. Biol. Med.,* 39, 1499–1511.

- El-Ghazali, I.; Saqrane, S.; Carvalho, A.; Ouahid, Y.; Del Campo, F. and Vasconcelos, V. 2010. Effects of the microcystin profile of a cyanobacterial bloom on growth and toxin accumulation in common carp (*Cyprinus carpio*) larvae. *J. Fish Biol*., 76(6), 1415-1430.

- El-Ghazali, I.; Saqrane, S.; Carvalho, A.; Ouahid, Y.; Oudra, Brahim; Del Campo, F. and Vasconcelos, V. 2009. Compensatory Growth Induced in Zebrafish Larvae after Pre-Exposure to a *Microcystis aeruginosa* Natural Bloom Extract Containing Microcystins. *Int. J. Mol. Sci.*, 10, 133-146.

- Falconer, I. 1991.Tumor promotion and liver injury caused by oral consumption of cyanobacteria. *Environ. Toxicol. Water Qual*., 6, 177–84.

- Falconer, I. 2007. Health effects associated with controlled exposures to cyanobacterial toxins. Proceedings of the interagency, international symposium on cyanobacterial harmful algal blooms (ISOC-HAB): State of the Science and Research Needs.

- Fawell, J.; Mitchell, R.; Everett, D. and Hill, R. 1999. The toxicity of cyanobacterial toxins in the mouse: I microcystin- LR. Hum. Exp. *Toxicol.*, 18 (3), 162–167.

- Ferrao-Filho Ada, S.; Kozlowsky-Suzuki, B. 2011. Cyanotoxins: Bioaccumulation and effects on aquatic animals. *Mar. Drugs*, 9, 2729–2772.

- Fischer, W. and Dietrich, D. 2000b. Pathological and biochemical characterization of microcystin induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.*, 164, 73–81.

- Fischer, W. and Dietrich, D. 2006a. Toxicity of the cyanobacterial cyclic heptapeptide toxins microcystin-LR and -RR in early life-stages of the African clawed frog (*Xenopus laevis*). *Aquat. Toxicol*., 49, 189–98.

- Fischer, W.; Hitzfeld, B.; Tencalla, F.; Eriksson, J.; Mikhailov, A. and Dietrich, D. 2000c. Microcystin-LR toxic dynamics, induced pathology, and immunohistochemical localization in livers of blue-green algae exposed rainbow trout (*Oncorhynchus mykiss*). *Toxicol., Sci*., 54, 365–73.

- Florczyk, M., Łakomiak, A., Woźny, M. and Brzuzan, P. 2014. Neurotoxicity of cyanobacterial toxins. *Environmental Biotechnology*, 10(1), 26-43.

- Fournie, J. and Courtney, L. 2002. Histopathological evidence of regeneration following hepatotoxic effects of the cyanotoxin microcystin-LR in the hardhead catfish and gulf killifish. *J. Aquat. Anim. Health*, 14(4), 273-280.

- Fournie, J.; Hawkins, W.; Krol, R. and Wolfe, M. 1996. *Preparation of whole small fish for histological evaluation*. In: Ostrander, GM., editor. *Techniques in Aquatic Toxicology*. Lewis Publishers; Boca Raton, Florida: p. 577-588.

- Fritsche, R.; Schwerte, T. and Pelster, B. 2000. Nitric oxide and vascular reactivity in developing zebrafish, *Danio rerio*. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., 279, R2200–R2207.

- Frostegard, A.; Courtois, S.; Ramisse, V.; Clerc, S.; Bernillon, D.; Le Gall, F.; Jeannin, P.; Nesme, X. and Simonet, P. 1999. Quantification of bias related to the extraction of DNA directly form soils. *Appl. Environ. Microbiol.,* 65, 5409-5420.

- Fujiki, H. and Suganuma, M. 2009. Carcinogenic aspects of protein phosphatase 1 and 2A inhibitors. *Progress in Molecular and Subcellular Biology*, 46, 221–254.

- Goel, A.; Gupta, M. and Aggarwal, R. 2014. Gut microbiota and liver disease. *J. Gastroenterol. Hepatol.,* 29, 1139-1148.

- Graham, J.; Loftin, K.; Meyer, M.; Ziegler, A. 2010. Cyanotoxin mixtures and taste-and-odor compounds in cyanobacterial blooms from the midwestern United States. *Comp. Biochem. Physiol. B: Environ. Sci. Technol*., 44, 7361–7368.

- Grizzle, J.; Putnam, M.; Fournie, J. and Couch, J. 1988. Microinjection of chemical carcinogens into small fish embryos: exocrine pancreatic neoplasm in Fundulusgrandis exposed to Nmethyl- N-nitro-N-nitrosoguanidine. *Dis. Aquat. Org*., 5, 101–105.

- Gupta, N., Pant, S., Vijayaraghavan, R. and Rao, P. 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice, *Toxicology*, 188(2-3), 285-296.

- Halliwell, B. and Gutteridge, J. editors. 2007. *Free radicals in biology and medicine*. Fourth ed. New York: Oxford University Press.

- Harada, K. 2004. Production of secondary metabolites by fresh water cyanobacteria. *Chem. Pharm. Bull*., 52 (8), 889–899.

- Harada, K.; Tsuji, K.; Watanabe, M.F. and Kondo, F. 1996. Stability of microcystins from cyanobacteria: III. Effect of pH and temperature. *Phycologia*., 35, 83– 88.

- Hou, J.; Li, L.; Xue, T.; Long, M.; Su, Y. and Wu, N. 2014. Damage and recovery of the ovary in female zebrafish i.p.-injected with MC-LR. *Aquat. Toxicol.*, 155, 110-118.

- Hudder, A.; Song, W.; O'Shea, K.; Walsh, P. 2007. Toxicogenomic evaluation of microcystin-LR treated with ultrasonic irradiation. *Toxicol. Appl. Pharm.*, 220, 357–364.

- Huynh-Delerme, C.; Edery, M.; Huet, H.; Puiseux-Dao, S.; Bernard, C. and Fontaine, J. 2005. Microcystin-LR and embryo-larval development of medaka fish, *Oryziaslatipes*. I. Effects on the digestive tract and associated systems. *Toxicon*., 46(1), 16-23.

- Imboden, M.; Goblet, C.; Korn, H. and Vriz, S. 1997. *Cytokeratin 8* is a suitable epidermal marker during zebrafish development. *CR Acad. Sci. III*, 320, 689–700.

- Ishida, K.; Okita, Y.; Matsuda, H.; Okino, T. and Murakami, M. 1999. Aeruginosins, protease inhibitors from the cyanobacterium *Microcystis aeruginosa*. *Tetrahedron*, 55 (36), 10971–10988.

- Ishii, H.; Nishijima, M. and Abe, T. 2004. Characterization of degradation process of cyanobacterial hepatotoxins by a gram-negative aerobic bacterium. *Water Res*., 38, 2667–2676.

- Ito, E.; Kondo, F. and harada, K. 2000. First report on the distribution of orally administrated microcystin-LR in mouse tissue using an immunostaining method. *Toxicon*, 38, 37-48.

- Jacoby, J.; Collier, D.; Welch, E.; Hardy, F. and Crayton, M. 2000. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Can. J. Fish Aquat. Sci*., 57, 231–240.

- Jacquet, C.; Thermes, V.; de Luze, A.; Puiseux-Dao, S.; Bernard, C.; Joly, J.; Bourrat, F. and Edery, M. 2004. Effects of microcystin-LR on development of medaka fish embryos (*Oryzias latipes*). *Toxicon*., 43(2), 141-147.

- Jaja-Chimedza, A., Gantar, M., Mayer, G. D., Gibbs, P. D. and Berry, J. P. 2012. Effects of cyanobacterial lipopolysaccharides from *microcystis* on glutathione-based detoxification pathways in the zebrafish (*Danio rerio*) embryo. *Toxins (Basel),* 4(6), 390-404.

- Ji, R.; Lua, X.; Li, X. and Pu, Y. 2009. Biological degradation of algae and microcystins by microbial enrichment on artificial media. *Ecological Engineering*, 35, 1584–1588.

- Jochimsen, E.; Carmichael, W.; An, J.; Cardo, D.; Cookson, S. and Holmes, C. 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N. Engl. J. Med*., 338, 873–8.

- Jos, Á.; Pichardo, S.; Prieto, A.; Repetto, G.; Vázquez, C. and Morenoa, I. 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. *Aquat. Toxicol*., 72, 261–71.

- Juhel, G.; Davenport, J.; O'Halloran, J.; Culloty, S.; O'Riordan, R.; James, K.; Furey, A. and Allis, O. 2006. Impacts of microcystins on the feeding behavior and energy balance of zebra mussels, Dreissena polymorpha: a bioenergetics approach. *Aquat. Toxicol*., 79, 391–400.

- Karickhoff, S.; Brown, D. and Scott, T. 1979. Sorption of hydrophobic pollutants on natural water sediments. *Water Research*, 13, 241-248.

- Kawai, H.; Arata, N. and Nakayasu, H. 2001. Three-dimensional distribution of astrocytes in zebrafish spinal cord. *Glia.*, 36, 406–413.

- Kotak, B.; Semalulu, S.; Fritz, D.; Prepas, E.; Hrudey, S. and Coppock, R. 1996. Hepatic and renal pathology of intraperitoneally administered microcystin-LR in rainbow trout (*Oncorhynchus mykiss*). *Toxicon.*, 34, 517–525.

- Kotak, B.; Zurawell, R.; Prepas, E. and Holmes, C. 1996. Microcystin-LR concentration in aquatic food web compartments from lakes of varying

trophic status. *Canada Journal of Fisheries and Aquatic Sciences*, 53, 1974–1985.

- Kozdroj, J. and Elsas J. 2001. Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. *J. Microbiol. Meth*., 43, 197-212.

- Kuiper-Goodman, T.; Falconer, I. and Fitzgerald J. 1999. *Human health aspects*. In: Chorus, I. and Bartram, J. editors. *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. London, UK: E & FN Spon. p. 113–53.

- Lahti, K.; Rapala, J.; Fardig, M.; Niemela, M. and Sivonen, K. 1997. Persistence of cyanobacterial hepatotoxin, microcystin-LR, in particulate material and dissolved in lake water. *Wat. Res*., 31(5), 1005-1012.

- Lance, E.; Josso, C.; Dietrich, D.; Ernst, B.; Paty, C.; Senger, F.; Bormans, M. and Gérard, C. 2010. Histopathology and microcystin distribution in *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacterial or dissolved microcystin-LR expo-sure. *Aquatic Toxicology*, 98, 211–220.

- Leão, J.; Geracitano, L.; Monserrat, J.; Amado, L. and Yunes, J. 2008. Microcystin-induced oxidative stress in *Laeonereis acuta* (Polychaeta, Nereididae). *Mar. Environ. Res.*, 66, 92–4.

- Lehtimaki, J.; Moisander, P.; Sivonen, K. and Kononen, K. 1997. Growth, nitrogen fixation, and nodularin production by two Baltic Sea cyanobacteria. *Appl. Environ. Microbiol.*, 63, 1647–1656. In: Chorus, I., Bartram, J. (Eds.), *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management. E & FN Spon.* An Imprint of Routledge, London and New York.

- Li, G.; Chen, J.; Xie, P.; Jiang, Y.; Wu, L. and Zhang, X. 2011. Protein expression profiling in the zebrafish (*Danio rerio*) embryos exposed to the microcystin-LR. *Proteomics*, 11, 2003–2018.

- Li, X.; Chung, I.; Kim, J. and Lee J. 2005. Oral exposure to *Microcystis* increases activity augmented antioxidant enzymes in the liver of Loach

(*Misgurnus mizolepis*) and has no effect on lipid peroxidation. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol*., 141(3), 292-296.

- Li, X.; Chung, I.; Kim, J. and Lee, J. 2004. Subchronic oral toxicity of microcystin in common carp *(Cyprinus carpio L.)* exposed to Microcystis under laboratory conditions. *Toxicon*., 44, 821–827.

- Li, X.; Liu, Y.; Song, L. and Liu, J. 2003. Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. *Toxicon*, 42, 85–9.

- Li, X.; Ma, J.; Fang, Q. and Li, Y. 2013. Transcription alterations of microRNAs, cytochrome P4501A1 and 3A65, and AhR and PXR in the liver of zebrafish exposed to crude microcystins. *Toxicon,* 73, 17–22.

- Li, X.; Wang, J.; Liang, J. and Liu, Y. 2007. Toxicity of microcystins in the isolated hepatocytes of common carp (*Cyprinus carpio L*.). *Ecotoxicol. Environ. Saf.,* 67, 447–451.

- Li, Y. and Han, X. 2012. Microcystin–LR causes cytotoxicity effects in rat testicular Sertoli cells. *Environ. Toxicol. Pharmacol*., 33, 318–326.

- Li, Y.; Chen, J.; Zhao, Q.; Pu, C.; Qiu, Z.; Zhang, R. and Shu, W. 2011. A Cross-Sectional Investigation of Chronic Exposure to Microcystin in Relationship to Childhood Liver Damage in the Three Gorges Reservoir Region, China. *Environ. Health Perspect*, 119, 1483–1488.

- Li, Y.; Sheng, J.; Sha, J. and Han, X. 2008. The toxic effects of microcystin- LR on the reproductive system of male rats in vivo and in vitro. *Reprod. Toxicol*., 26, 239–245.

- Liang, G.; Xie, P.; Chen, J. and Yu, T. 2011. Comparative studies on the pH dependence of D_{OW} of microcystin-RR and -LR using LC-MS. *The Scientific World J.*, 11, 20–26.

- Lin, J.; Chen, J.; He, J.; Chen, J.; Yan, Q.; Zhou, J. and Xie, P. 2015. Effects of microcystin-LR on bacterial and fungal functional genes profile in rat gut. *Toxicon.*, 96, 50-56.

- Linney, E.; Upchurch, L. and Donerly, S. 2004. Zebrafish as a Neurotoxicological Model. *Neurotoxicol. Teratol*., 26, 709–718.

- Liu, J. and Sun, Y. 2015. The role of PP2A-associated proteins and signal pathways in microcystin-LR toxicity. *Toxicology Letters*, 236: 1–7.

- Liu, W.; Qiao, Q.; Chen, Y.; Wu, K. and Zhang, X. 2014. Microcystin-LR exposure to adult zebrafish (*Danio rerio*) leads to growth inhibition and immune dysfunction in F1 offspring, a parental transmission effect of toxicity. *Aquatic Toxicology*, 155, 360–367.

- Liu, Y.; Song, L.; Li X. and Liu, T. 2002. The toxic effects of microcystin-LR on embryo-larval and juvenile development of loach, *Misguruns mizolepis* Günther. *Toxicon.*, 40(4), 395-399.

- Liu, Y.; Xie, P.; Qiu, T.; Li, H. and Li, G. 2010. Microcystin extracts induce ultrastructure damage and biochemical disturbance in male rabbit testis. *Environ. Toxicol.*, 25, 9–17.

- Luckas, B.; Dahlmann, J.; Erler, K.; Gerdts, G.; Wasmund, N. and Hummert, C. 2005. Overview of key phytoplankton toxins and their recent occurrence in the North and Baltic seas. *Environ. Toxicol*., 20(1), 1-17.

- Mackay D. 1982. Correlation of bioconcentration factors. *Environmental Science and Technology*, 16: 274-278.

- MacKintosh, C.; Beattie, K. A.; Klumpp, S.; Cohen, P. and Codd, G. 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2a from both mammals and higher plants. *F.E.B.S. Lett*., 264, 187–92.

- Magalha`es, F.; Soares, R. and Azvedo, S. 2001. Microcystin contamination in fish from Jacarepagua´ Lagoon (Rio de Janeiro, Brazil): ecological implication and human health risk. *Toxicon,* 39, 1077–1085.

- Malbrouck, C.; Kestemont, P. 2006. Effects of microcystins on fish. Environ. *Toxicol. Chem.*, 25 (1), 72–86.

- Malbrouck, C.; Trausch, G.; Devos, P. and Kestemont P. 2003. Hepatic accumulation and effects of microcystin-LR on juvenile goldfish (*Carassius auratus L.*). *Comp. Biochem. Physiol. C. Toxicol. Pharmacol*., 135(1), 39-48.

- Mares, J.; Palikova, M.; Kopp, R.; Navratil, S. and Pikula, J. 2009. Changes in the nutritional parameters of muscles of the common carp (*Cyprinus carpio*) and the silver carp (*Hypophthalmic hthysmolitrix*) following environmental exposure to cyanobacterial water bloom. *Aquac. Res*., 40(2), 148-156.

- Marin, M. and Matozzo, V. 2004. Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. *Marine Pollution Bulletin*, 48, 835–839.

- Merel, S., Walker, D., Chicana, R., Snyder, S., Baures, E. and Thomas, O. 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environ Int.*, 59, 303-27.

- Metcallfe, C. and Sonstegard, R. 1985. Oil refinery effluents: evidence of cocarcinogenic activity in the trout embryo microinjection assay. *J. Natl. Cancer Inst*., 75, 1091–1097.

- Mezhoud, K.; Bauchet, A.; Chateau-Joubert, S.; Praseuth, D.; Marie, A.; Francois, J.; Fontaine, J.; Jaeg, J.; Cravedi, J.; Puiseux-Dao, S. and Edery, M. 2008. Proteomic and phosphoproteomic analysis of cellular responses in medaka fish (*Oryzias latipes)* following oral gavage with microcystin-LR. *Toxicon*., 51, 1431–1439.

- Miller, M. and Fallow, H. 2001. Degradation of cyanobacterial hepatotoxins in batch experiments. *Water Sci. Technol*., 43(12): 229–232.

- Mohamed, Z.; Carmichael, W. and Hussein, A. 2003. Estimation of microcystins in the freshwater fish *Oreochromis niloticus* in an Egyptian fish farm containing a Microcystis bloom. *Environmental Toxicology*, 18, 137–141.

- Morena, I.; Pichardo, S.; Jos, A.; Gomez-Amores, L.; Mate, A.; Vazquez, C. and Camean, A. 2005. Antioxidant enzyme activity and lipid peroxidation

in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. *Toxicon*., 45, 395–402.

- Mur, L.; Skulberg, M. and Utkilen, H. 1999. *Cyanobacteria in the environment*. In: Chorus I, Bartram J, editors. *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. London, UK: E & FN Spon. p. 15–40.

- Nebert, D.; Dalton, T.; Okey, A. and Gonzalez, F. 2004. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J. Biol. Chem.,* 279, 23847–23850.

- Nelson, D. 2003. Comparison of P450s from human and fugu: 420 Million years of vertebrate P450 evolution. *Arch. Biochem. Biophys.,* 409, 18–24.

- Nicholson, B.; Rositano, J. and Burch, M. 1994. Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *War. Res*., 28, 1297-1303.

- Niedermeyer, T.; Daily, A.; Swiatecka-Hagenbruch, M. and Moscow, J. 2014. Selectivity and Potency of Microcystin Congeners against OATP1B1 and OATP1B3 Expressing Cancer Cells. *PLoS ONE,* 9(3).

- NOAA. 2014. Research continues on the causes of harmful algal blooms. [Online]. Available from: http://oceanservice.noaa.gov/facts/why_habs.html (Accessed 26/1/2015).

- Oberemm, A.; Becker, J.; Codd, G. and Steinberg, C. 1999. Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. *Environ. Toxicol.,* 14 (1), 77–88.

- Oberemm, A.; Fastner, J.; Steinberg, C. 1997. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (*Danio rerio*). *Water Res*., 31 (11), 2918–2921.

- Orr, P. and Jones, G. 1998. Relationship between microcystin production and cell division rates in nitrogen-limited Microcystis aeruginosa cultures. *Limnol. Oceanogr*., 43, 1604–1614.

- Ozawa, K.; Yokoyama, A.; Ishikawa, K.; Kumagai, M., Watanabe, M. and Park, H. 2003. Accumulation and depuration of microcystin produced by the

cyanobacterium *Microcystis* in a freshwater snail. *Limnology*, 4, 131–138.

- Oziol, L. and Bouaïcha, N. 2010. First evidence of estrogenic potential of the cyanobacterial heptotoxins the nodularin-R and the microcystin-LR in cultured mammalian cells. *J. Hazard. Mater*., 174:(1-3), 610–615.

- Pakdel, F.; Féon, S.; Gac, F.; Menn, F. and Valotaire, Y. 1991. In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. *Molecular and Cellular Endocrinology*, 75:205–212.

- Palikova, M.; Krejci, R.; Hilscherova, K.; Babica, P.; Navratil, S.; Kopp, R. and Blaha, L. 2007. Effect of different cyanobacterial biomasses and their fractions with variable microcystin content on embryonal development of carp (*Cyprinus carpio L*.). *Aquat. Toxicol*., 81 (3), 312–318.

- Palus, J.; Dziubałtowska, E.; Sta´nczyk, M.; Lewi´nska, D.; Mankiewicz-Boczek, J.; Izy-dorczyk, K.; Bonisławska, A.; Jurczak, T.; Zalewski, M. and Wasowicz, W. 2007. Biomonitoring of cyanobacterial blooms in polish water reservoir and the cytotoxicity and genotoxicity of Selected Cyanobacterial Extracts. *Int. J. Occup. Med.Environ*., 20, 48–65.

- Papadimitriou, T.; Armeni, E.; Stalikas, C.; Kagalou, I. and Leonardos, I. 2012. Detection of microcystins in pamvotis lake water and assessment of cyanobacterial bloom toxicity. *Environ. Monit. Assess*., 184, 3043–3052.

- Papadimitriou, T.; Kagalou, I. and Leonardos, I. 2012. Seasonally accumulation of microcystins in the various tissues of an endemic and protected fish species (*Rutilus panosi*) with different sizes. *CLEAN-Soil. Air. Water*, 40, 402–407.

- Papadimitriou, T.; Kagalou, I.; Bacopoulos, V. and Leonardos, I. 2009. Accumulation of microcystins in water and fish tissues: an estimation of risks associated with microcystins in most of the Greek lakes. *Environmental Toxicology*, 25, 418–427.

- Park, C.; Aoki, J.; Lee, J.; Nagae, M.; Lee, Y.; Sakakura, Y.; Hagiwara, A. and Soyano, K. 2010. The effects of 17 _-estradiol on various reproductive parameters in the hermaphrodite fish Kryptolebias marmoratus. *Aquatic Toxicology*, 96, 273–279.

- Park, H.; Watanabe, M.; Harada, K.; Suzuki, M. Hayashi, H. and Okino, T. 1993. Seasonal variations of Microcystis species and toxic heptapeptide microcystins in Lake Suwa. *Environ. Toxicol. Water Qual.*, 8, 425-435.

- Pavagadhi, S. and Balasubramanian, R. 2013a. Toxicological evaluation of microcystins in aquatic fish species: Current knowledge and future directions. *Aquat. Toxicol*., 142–143, 1–16.

- Pavagadhi, S.; Gong, Z. and Balasubramanian, R. 2013b. Toxicological implications of microcystins for zebrafish embryos in the presence of other environmental pollutants. *Environ. Toxicol., Chem*., 32, 1574–1581.

- Pavagadhi, S.; Gongc, Z.; Handed, M.; Dionysioue, D.; De la Cruzf, A. and Balasubramanian, R. 2012. Biochemical response of diverse organs in adult *Danio rerio* (zebrafish) exposed to sub-lethal concentrations of microcystin-LR and microcystin-RR: A balneation study. *Aquatic Toxicology*, 109, 1-10.

- Pérez, S. and Aga, D. 2005. Recent advances in the sample preparation, liquid chromatography tandem mass spectrometric analysis and environmental fate of microcystins in water. *Trends Anal. Chem*., 24(7), 658–670.

- Peterson, J.; Garges, S.; Giovanni, M.; McInnes, P.; Wang, L. and Schloss, J. 2009. The NIH Human Microbiome Project. *Genome Res*., 19, 2317–2323.

- Pflugmacher, S.; Wiegand, C.; Oberemm, A.; Beattie, K.; Krause, E.; Codd, G. and Steinberg, C. 1998. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of detoxication. *Biochim. Biophys. Acta*., 1425, 527–533.

- Pinho, G.; Moura, D.; Maciel, F.; Bianchini, A.; Yunes, J. and Proença, L. 2005. Antioxidant responses and oxidative stress after microcystin exposure

in the hepatopancreas of an estuarine crab species. *Ecotoxicol. Environ. Saf.*, 61, 353–60.

- Pouria, S.; de Andrade, A.; Barbosa, J.; Cavalcanti, R.; Barreto, V. and Ward, C. 1998. Fatal microcystin intoxication in hemodialysis unit in Caruaru, Brazil. *Lancet*, 352, 21–6.

- Prieto, A.; Pichardo, S.; Jos, A.; Moreno, I. and Cameán, A. 2007. Time-dependent oxidative stress responses after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (*Oreochromis niloticus*) under laboratory conditions. *Aquat. Toxicol*., 84, 337–45.

- Qiao, Q.; Liu, W.; Wu, K.; Song, T.; Hu, J.; Huang, X.; Wen, J.; Chen, L. and Zhang, X. 2013. Female zebrafish (*Danio rerio*) are more vulnerable than males to microcystin-LR exposure, without exhibiting estrogenic effects. *Aquatic Toxicology*, 142– 143, 272– 282.

- R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

- Rabergh, C.; Bylund, G. and Eriksson, J. 1991. Histopathological effects of microcystin-LR a cyclic peptide toxin from the cyanobacterium blue-green alga Microcystis-aeruginosa on common carp *Cyprinus-carpio L. Aquat. Toxicol.* 20, 131–146.

- Ranjard, L.; Poly, F. and Nazaret, S. 2000. Monitoring complex bacterial communities using culture independent molecular techniques: application to soil environment. *Res. Microbiol*., 151, 167-177.

- Rapala, J.; Berg, K.; Lyra, C.; Niemi, R.; Manz, W.; Suomalainen, S.; Paulin, L. and Lahti, K. 2005. Paucibactertoxinivorans gen. nov., sp. nov., a bacterium that degrades cyclic cyanobacterial hepatotoxins microcystins and nodularin. Int. *J. Syst. Evol. Microbiol*., 55, 1563–1568.

- Rapala, J.; Sivonen, K.; Lyra, C. and Niemala, S. 1997. Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Appl. Environ. Microbiol*., 63, 2206–2212.

- Raziuddin, S.; Siegelman, H.; Tornabene, T. 1983. Lipopolysaccharides of the cyanobacterium Microcystis aeruginosa. *Eur. J. Biochem.*, 137 (1-2), 333–336.

- Reinardy, H.; Dharamshi, J.; Awadhesh N. and Henry, T. 2013. Changes in expression profiles of genes associated with DNA repair following induction of DNA damage in larval zebrafish *Danio rerio*. *Mutagenesis*, 28(5), 1–8.

- Rivasseau, C.; Martins, S. and Hennion, M. 1998. Determination of some physicochemical parameters of microcystins (cyanobacterial toxins) and trace level analysis in environmental samples using liquid chromatography. *Journal of Chromatography A*, 799 (1-2), 1555-169.

- Rocke, J.; Lees, J.; Packham, I. and Chico, T. 2009. The zebrafish as a novel tool for cardiovascular drug discovery. *Recent Pat. Cardiovasc. Drug Discov*., 4, 1–5.

- Roeselers, G.; Mittge, E.; Stephens, W.; Parichy, D.; Cavanaugh, C.; Guillemin, K. and Raw, J. 2011. Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5, 1595–1608.

- Rogers, E. D., Henry, T. B., Twiner, M. J., Gouffon, J. S., McPherson, J. T., Boyer, G. L., Sayler, G. S. and Wilhelm, S. W. 2011. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and *Microcystis* reveals endocrine disrupting effects of Cyanobacteria. *Environ. Sci. Technol*., 45(5), 1962-9.

- Rohrlack, T.; Christoffersen, K.; Hansen, P. E.; Zhang, W.; Czarnecki, O.; Henning, M.; Fastner, J.; Erhard, M.; Neilan, B. A. and Kaebernick, M. 2003. Isolation, characterization, and quantitative analysis of microviridin J, a new *Microcystis* metabolite toxic to Daphnia. *J. Chem. Ecol*., 29 (8), 1757–1770.

- Rosa, C.; de Souza, M.; Yunes, J.; Proença, L.; Nery, L. and Monserrat, J. 2005. Cyanobacterial blooms in estuarine ecosystems: characteristics and effects on *Laeonereis acuta* (Polychaeta, Nereididae). *Mar. Pollut. Bull.*, 50, 956–64.

- Runnegar, M.; Kong, S. and Berndt, N. 1993. Protein phosphates inhibition and in-vivo hepatotoxicity of microcystin. *Am. Physiol. Soc*., 265, G224–G231.

- Schmidt, J.R.; Wilhelm, S. and Boyer, G. 2014. The fate of microcystins in the environment and challenges for monitoring. *Toxins,* 6, 3354–3387.

- Scott, W.E. 1991. Occurrence and significance of toxic Cyanobacteria in Southern Africa. *Wat. Sci. Techno*., 23, 175-180.

- Seki, H.; Takahashi, M.; Hara, Y. and Ichimura, S. 1980. Dynamics of dissolved oxygen during algal bloom in lake Kasumigaura, Japan. *Water Res.*, 14, 179–83.

- Singh, D.; Kashyap, A.; Pandey, R. and Saini, K. 2011. Novel advances in cytochrome P450 research. *Drug Discov. Today,* 16, 793–799.

- Sivonen, K. and Jones, G. 1999. *Cyanobacterial toxins*. In: Chorus, I. and Bartram, J. (eds). *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. London, UK: E & FN Spon. pp. 41–111.

- Sivonen, K. 1990. Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by Oscillatoria agardhii strains. *Appl. Environ. Microbiol*., 56, 2658–2666.

- Skrajnowska, D., Bobrowska-Korczak, B., Tokarz, A., Bialek, S., Jezierska, E. and Makowska, J. 2013. Copper and resveratrol attenuates serum catalase, glutathione peroxidase, and element values in rats with DMBA-induced mammary carcinogenesis. *Biol. Trace Elem. Res.*, 156(1-3), 271-8.

- Smith, J.; Boyer, G. and Zimba, P. 2008. A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture*, 280 (1-4), 5–20.

- Soares, R.; Magalhaes, V. and Azevedo, S. 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions. *Aquat. Toxicol*., 70(1), 1-10.

- Spitsbergen, J. and Kent, M. 2003. The State of the Art of the Zebrafish Model for Toxicology and Toxicologic Pathology Research—Advantages and Current Limitations. *Toxicol. Pathol*., 31: 62–87.

- Strack, S.; Cribbs, J. and Gomez, L. 2004. Critical role for protein phosphatase 2A heterotrimers in mammalian cell survival. *J. Biol. Chem.*, 279: 47732–47739.

- Strack, S.; Cribbs, J. and Gomez, L. 2004. Critical role for protein phosphatase 2A heterotrimers in mammalian cell survival. *J. Biol. Chem.*, 279, 47732–47739.

- Sumpter, J. and Jobling, S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*, 103:173.

- Sun, Y.; Zheng, Q.; Sun, Y.; Huang, P.; Guo, Z. and Xu, L. 2014. Microcystin-LR induces protein phosphatase 2A alteration in a human liver cell line. *Environ. Toxicol*., 29: 1236–1244.

- Takenaka, S. and Watanabe, M. 1997. Microcystin LR degradation by *Pseudomonas aeruginosa* alkaline protease. *Chemosphere*, 34 (4), 749–757.

- Tencalla, F. and Dietrich, D. 1997. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). *Toxicon*., 35(4), 583-595.

- Tencalla, F.; Dietrich, D. and Schlatter, C. 1994. Toxicity of Microcystis aeruginosa peptide toxin to yearling rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 30, 215–224.

- Tiedje, J.; Asuming-Brempong, S.; Nusslein, K.; Marsh, T. and Flynn, S. 1999. Opening the black box of soil microbial diversity. *Appl. Soil Ecol*., 13, 109-122.

- Trinchet, I.; Djediat, C.; Huet, H.; Dao, S. and Edery, M. 2011. Pathological modifications following sub-chronic exposure of medaka fish (*Oryzias latipes*) to microcystin-LR. *Reproductive Toxicology*, 32: 329–340.

- Tsai, C.; Tseng, J.; Lin, S.; Chang, C.; Wu, J.; Horng, J. and Tsay, H. 2001. Primary structure and developmental expression of zebrafish sodium channel Na(v)1.6 during neurogenesis. *DNA Cell Biol.*, 20, 249–255.

- Tsuji, K.; Naito, S.; Kondo, F.; Ishikawa, N.; Watanabe, M.; Suzuki, M. and Harada, K. 1994. Stability of microcystins from cyanobacteria: effect of light on decomposition and isomerization. *Environ. Sci. Technol*., 28, 173-177.

- Tsuji, K.; Watanuki, T.; Kondo, F.; Watanab, M.; Suzuki, S.; Nakazawa, H.; Suzuki, M.; Uchida, H. and Harada, K. 1995. Stability of microcystin from cyanobacteria –II. Effects of UV light on decomposition and isomerization. *Toxicon*., 33 (12), 1619-1631.

- Tsuji, K.; Watanuki, T.; Kondo, F.; Watanabe, M.; Kazawa, H.; Suzuki, M.; Uchida, H. and Harada, K. 1997. Stability of microcystins from cyanobacteria-IV. Effects of chlorination on decomposition. *Toxicon*., 35(7), 1033-1041.

- Tzima, E.; Serifi, I.; Tsikari, I.; Alzualde, A.; Leonardos, I.; and Papamarcaki, T. 2017. Transcriptional and Behavioral Responses of Zebrafish Larvae to Microcystin-LR Exposure. *Int. J. Mol. Sci.*, 18, 365-379.

- Van Nassauw L. and Harrisson, F. 1991. Callebaut M. Localization of smooth-muscle markers in the ovaries of some ectothermic vertebrates. *Anat. Rec.*, 229, 439–446.

- Van Raamsdonk, W.; Tekronnie, G.; Pool, C. and van de Laarse, W. 1980. An immune histochemical and enzymic characterization of the muscle fibers in myotomal muscle of the teleost *Brachydanio rerio*, Hamilton-Buchanan. *Acta. Histochem*., 67, 200–216.

- Villamizar, N.; Ribas, L.; Piferrer, F.; Vera, L. and Sanchez-Vazquez, F. 2012. Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. *PloS One,* 7, 521-531.

- Vinagre, T.; Alciati, J.; Regoli, F.; Bocchetti, R.; Yunes, J. and Bianchini, A. 2003. Effect of hepatotoxins (microcystin) on ion-regulation and antioxidant system in gills of *Chasmagnathus granulatus* (Decapoda, Grapsidae). *Comp. Biochem. Physiol. C.*, 135, 67–75.

- Votto, A.; Renon, V.; Yunes, J.; Rumjanek, V.; Marques, C. and Moura, N. 2007. Sensitivity to microcystins: a comparative study in human cell lines with and without multidrug resistance phenotype. *Cell Biol. Int.,* 31, 1359–66.

- Wallace, K. and Pack, M., 2003. Unique and conserved aspects of gut development in zebrafish. *Dev. Biol*., 255: 12-29.

- Wallace, K.; Akhter, S.; Smith, E.; Lorent, K. and Pack, M., 2005. Intestinal growth and differentiation in zebrafish. *Mech. Dev.*, 122, 157-173.

- Wang, H.; Tan, J.; Emelyanov, A.; Korzh, V. and Gong, Z. 2005. Hepatic and extra-hepatic expression of vitellogenin genes in the zebrafish, *Danio rerio. Gene*., 356, 91–100.

- Wang, M.; Chan, L.; Si, M.; Hong, H. and Wang., D. 2010. Proteomic Analysis of Hepatic Tissue of Zebrafish (*Danio rerio*) Experimentally Exposed to Chronic Microcystin-LR. *Toxicological Sciences*, 113(1), 60–69.

- Wang, P.; Chien, M.; Wu, F.; Chou, H. and Lee, S. 2005. Inhibition of embryonic development by microcystin-LR in zebrafish. *Danio rerio*. *Toxicon.,* 45 (3), 303–308.

- Watanabe, M.; Park, H.; Kondo, F.; Harada, K.; Hayashi, H. and Okino, T. 1997. Identification and estimation of microcystins in freshwater mussels. *Natural Toxins*, 5, 31–35.

- WHO. 1999. *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management*. Edited by Ingrid Chorus and Jamie Bartram. Geneva.

- WHO. 2003. *Cyanobacterial Toxins: Microcystin-LR in Drinking-Water*. Background Document for Development of WHO Guidelines for Drinking- Water Quality. Geneva.

- Wiegand, C.; Pflugmacher, S.; Oberemm, A.; Meems, N.; Beattie, K. A.; Steinberg, C. and Codd, G. 1999. Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*). *Environ. Toxicol*., 14 (1), 89–95.

- Wu, J.; Shao, S.; Zhou, F.; Wen, S.; Chen, F. and Han, X. 2014. Reproductive toxicity on female mice induced by microcystin-LR. *Environmental Toxicology and Pharmacology*, 37, 1–6.

- Wullimann, M. and Rink, E. 2001. Detailed immunohistology of Pax6 protein and tyrosine hydroxylase in the early zebrafish brain suggests role of *Pax6* gene in development of dopaminergic diencephalic neurons. *Brain Res. Dev*., 131, 173–191.

- Xing, Y.; Xu, Y.; Chen, Y.; Jeffrey, P.; Chao, Y.; Lin, Z.; Li, Z.; Strack, S.; Stock, J.B. and Shi, Y. 2006. Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell*, 127, 341–353.

- Xiong, Q.; Xie, P.; Li, H.; Hao, L. and Li, G. 2010. Acute effects of microcystins exposure on the transcription of antioxidant enzyme genes in three organs (liver, kidney, and testis) of male wistar rats. *J. Biochem. Mol. Toxicol.*, 24, 361–367.

- Xiong, Q.; Xie, P.; Li, Y.; Hao, L.; Li, G.; Qiu, T. and Liu, Y. 2009. Involvement of Fas/FasL system in apoptotic signaling in testicular germ cells of male Wistar rats injected i.v. with microcystins. *Toxicon*., 54, 1–7.

- Yamaki, H.; Sitachitta, N.; Sano, T. and Kaya, K. 2005. Two new chymotrypsin inhibitors isolated from the cyanobacterium *Microcystis aeruginosa* NIES-88. *J. Nat. Prod.*, 68 (1), 14–18.

- Yang, Y., Tomkovich, S. and Jobin, C. 2014. Could a swimming creature inform us on intestinal diseases? Lessons from zebrafish. *Inflamm. Bowel Dis*., 20, 956-966.

- Yelon, D.; Horne, S. and Stainier, D. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev. Biol.,* 214, 23–37.

- Yuan, M.; Carmichael, W. and Hilborn, E. 1996. Microcystin analysis in human sera and liver from human fatalities in Caruaru, Brazil. *Toxicon.,* 48:627–40.

- Zegura, B.; Sedmak, B. and Filipic M. 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. *Toxicon,* 41, 41–8.

- Zegura, B.; Zajc, I.; Lah, T. and Filipic, M. 2008. Patterns of microcystin-LR induced alteration of the expression of genes involved in response to DNA damage and apoptosis. *Toxicon,* 51, 615–23.

- Zhang, B.; Liu, Y. and Li, X. 2015. Alteration in the Expression of Cytochrome P450s (CYP1A1, CYP2E1, and CYP3A11) in the Liver of Mouse Induced by Microcystin-LR. *Toxins,* 7, 1102-1115.

- Zhang, H.; Zhang, J.; Chen, Y. and Zhu, Y. 2007. Influence of intracellular Ca2+, mitochondria membrane potential, reactive oxygen species, and intracellular ATP on the mechanism of microcystin-LR induced apoptosis in Carassius auratus lymphocytes in vitro. *Environ Toxicol.*, 22, 559–64.

- Zhang, X.; Xie, P.; Wang, W.; Li, D.; Li, L.; Tang, R.; Lei, H. and Shi, Z. 2008. Dose-dependent effects of extracted microcystins on embryonic development, larval growth and histopathological changes of southern catfish (*Silurus meridionalis*). *Toxicon.*, 51, 449–456.

- Zhao, S.; Xie, P.; Li, G.; Chen, J. and Cai, Y. 2012. The proteomic study on cellular responses of the testes of zebrafish (*Danio rerio*) exposed to microcystin- RR. *Proteomics*, 12, 300–312.

- Zhao, Y.; Xie, L. and Yan, Y. 2015. Microcystin-LR impairs zebrafish reproduction by affecting oogenesis and endocrine system. *Chemosphere*, 120, 115-22.

- Zurawell, R.; Chen, H.; Burke, J. and Prepas, E. 2005. Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. *J. Toxicol. Environ. Health*, 8, 1–37. In: Lance, E.; Brient, L.; Bormans, M. and Gerard, C. (Eds.), 2006. Interactions between cyanobacteria and Gastropods I. Ingestion of toxic Planktothrixagardhii by *Lymnaea stagnalis* and kinetics of microcystin bioaccumulation and detoxification. *Aquat. Toxicol*., 79, 140–148.

- Zurawell, R.; Kotak, B. and Prepas, E. 1999. Influence of lake trophic status on the occurrence of microcystin-LR in the tissue of pulmonate snails. *Freshwater Biology*, 42, 707–718.